



## INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

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<b>(21) International Application Number:</b> PCT/US95/04063 <b>(22) International Filing Date:</b> 30 March 1995 (30.03.95)  <b>(30) Priority Data:</b> 08/219,842 30 March 1994 (30.03.94) US 08/397,808 3 March 1995 (03.03.95) US  <b>(60) Parent Applications or Grants</b> (63) Related by Continuation US 08/219,842 (CIP) Filed on 30 March 1994 (30.03.94) US 08/397,808 (CIP) Filed on 3 March 1995 (03.03.95)  <b>(71) Applicant (for all designated States except US):</b> APPLIED GENETICS, INC. [US/US]; Suite A, 11494 Sorrento Valley Road, San Diego, CA 92121 (US).  <b>(72) Inventors; and</b> <b>(75) Inventors/Applicants (for US only):</b> HERRNSTADT, Corinna [DE/US]; 3628 Ruette DeVille, San Diego, CA 92130 (US). PARKER, William, Davis [US/US]; 1680 Old Ballard Road, Charlottesville, VA 22901 (US). DAVIS, Robert, E.		[US/US]; 13272 Glen Cliff Way, San Diego, CA 92130 (US). MILLER, Scott, William [US/US]; Apartment #08, 781 S. Nardo Road, Solana Beach, CA 92075 (US).  <b>(74) Agents:</b> MacWRIGHT, Robert, S. et al.; Kenyon & Kenyon, One Broadway, New York, NY 10004 (US).  <b>(81) Designated States:</b> AM, AU, BB, BG, BR, BY, CA, CN, CZ, EE, FI, GE, HU, IS, JP, KE, KG, KP, KR, KZ, LK, LR, LT, LV, MD, MG, MN, MW, MX, NO, NZ, PL, RO, RU, SD, SG, SI, SK, TJ, TT, UA, UG, US, UZ, VN, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG), ARIPO patent (KE, MW, SD, SZ, UG).  <b>Published</b> <i>With international search report.</i>
<b>(54) Title:</b> DIAGNOSIS, THERAPY AND CELLULAR AND ANIMAL MODELS FOR DISEASES ASSOCIATED WITH MITOCHONDRIAL DEFECTS  <b>(57) Abstract</b>  <p>The present invention relates to genetic mutations in mitochondrial cytochrome c oxidase genes that segregate with Alzheimer's disease (AD), diabetes mellitus, Parkinson's disease and other diseases of mitochondrial origin. The invention provides methods for detecting these mutations, either before or after the onset of clinical symptoms. The invention further provides treatment of cytochrome c oxidase dysfunction. Cybrid cell lines which have utility as model systems for the study of disorders that are associated with mitochondrial defects are also described. The cybrids are constructed by treating immortal cell lines with an agent that irreversibly disables mitochondrial electron transport, and then transfecting the cells with mitochondria isolated from diseased tissue samples. One such cybrid was constructed using neuroblastoma cells and mitochondria from a patient suffering from Alzheimer's Disease. Methods for using such cybrids for screening drugs and therapies for utility in treating such disorders are also provided. In addition, cybrid animals, methods of producing them, and methods of using them in drug and therapy screening are also provided.</p>		

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DIAGNOSIS, THERAPY AND CELLULAR AND ANIMAL MODELS FOR  
DISEASES ASSOCIATED WITH MITOCHONDRIAL DEFECTS

This application is a continuation-in-part of co-  
pending application Serial No. 08/397,808, filed on  
March 3, 1995 for CELLULAR AND ANIMAL MODELS FOR  
DISEASES ASSOCIATED WITH MITOCHONDRIAL DEFECTS, of co-  
5 pending application Serial No. \_\_\_\_\_, filed on March 30,  
1995 for MITOCHONDRIAL DNA MUTATIONS THAT SEGREGATE WITH  
LATE ONSET DIABETES MELLITUS, of co-pending application  
Serial No. \_\_\_\_\_ filed on March 30, 1995 for DIAGNOSTIC  
AND THERAPEUTIC COMPOSITIONS FOR ALZHEIMER'S DISEASE,  
10 and of co-pending application Serial No. 08/219,842  
filed on March 30, 1994 for DIAGNOSTIC AND THERAPEUTIC  
COMPOSITIONS FOR ALZHEIMER'S DISEASE, all of which are  
incorporated herein by reference.

15 FIELD OF THE INVENTION

The present invention relates to the diagnosis and  
treatment of diseases of mitochondrial origin. More  
specifically, the invention relates to detecting genetic  
mutations in mitochondrial cytochrome c oxidase genes as  
20 a means for diagnosing Alzheimer's disease and diabetes  
mellitus, and suppressing these same mutations or the

effects of these mutations in the treatment of Alzheimer's disease and diabetes mellitus. The present invention also relates generally to model systems for diseases that involve defects in the function of mitochondria, where those defects arise from defects in the genes of those mitochondria. The invention also relates to the use of these model systems for screening drugs and evaluating the efficacy of treatments for those diseases. It also relates to the use of these model systems for the diagnosis of such diseases.

#### BACKGROUND OF THE INVENTION

Alzheimer's disease (AD) is a progressive neurodegenerative disorder characterized by loss and/or atrophy of neurons in discrete regions of the brain, accompanied by extracellular deposits of  $\beta$ -amyloid and the intracellular accumulation of neurofibrillary tangles. It is a uniquely human disease, affecting over 13 million people worldwide. It is also a uniquely tragic disease. Many individuals who have lived normal, productive lives are slowly stricken with AD as they grow older, and the disease gradually robs them of their memory and other mental faculties. Eventually, they even cease to recognize family and loved ones, and they often require continuous care until their eventual death.

Alzheimer's disease is incurable and untreatable, except symptomatically. Persons suffering from Alzheimer's disease may have one of two forms of this disease: "familial" AD or "sporadic" AD.

Familial Alzheimer's disease accounts for only about 5 to 10% of all Alzheimer's cases and has an unusually early-onset, generally before the age of fifty. Familial AD is inherited and follows conventional patterns of mendelian inheritance. This form of AD has been linked to nuclear chromosomal abnormalities.

In contrast, the second form of Alzheimer's disease, sporadic AD, is a late-onset disease which is neither inherited nor caused by nuclear chromosomal abnormalities. This late onset form of the disease is  
5 the more common type of Alzheimer's disease and is believed to account for approximately 90 to 95 % of all Alzheimer's cases.

To date, the diagnosis of probable Alzheimer's disease is only by clinical observation and is a  
10 diagnosis of exclusion. Unfortunately, definitive diagnosis can be accomplished only by pathological examination at autopsy. While attempts have been made to diagnose Alzheimer's disease by identifying differences in certain biological markers, including protease nexin  
15 II and apolipoprotein E alleles, this approach has not been successful. Incomplete penetrance in AD patients or crossover into normal or other disease populations makes identification of biological markers an unreliable method of diagnosis. Moreover, current therapies in  
20 clinical evaluation are designed to treat the symptoms of the disease and not impact the underlying pathology of AD. These therapies include Cognex, E2020, and other similar agents known in the field. However, since the primary etiologic events in AD are not yet known in the  
25 art, rational therapies have not been designed.

Parkinson's disease (PD) is a progressive neurodegenerative disorder characterized by the loss and/or atrophy of dopamine-containing neurons in the *pars compacta* of the *substantia nigra* of the brain.  
30 Like AD, PD also afflicts the elderly. It is characterized by bradykinesia (slow movement), rigidity and a resting tremor. Although L-Dopa treatment reduces tremors in most patients for a while, ultimately the tremors become more and more uncontrollable, making it  
35 difficult or impossible for patients to even feed themselves or meet their own basic hygiene needs.

Diabetes mellitus is a common degenerative disease affecting 5 to 10 percent of the population in developed countries. It is a heterogenous disorder with a strong genetic component, with indications that maternal  
5 heredity is an important factor. Monozygotic twins are highly concordant and there is a high incidence of the disease among first degree relatives of affected individuals. Maternal heredity reportedly contributes a propensity for developing diabetes mellitus. Alcolado,  
10 J.C. and Alcolado, R., Br. Med. J. 302: 1178-1180 (1991); Reny., S.L., International J. Epidem. 23: 886-890 (1994).

Studies have shown that diabetes mellitus may be preceded by or associated with certain related  
15 disorders. For example, it is estimated that forty million individuals in the U.S. suffer from late onset impaired glucose tolerance (IGT). IGT patients fail to respond to glucose with increased insulin secretion. A small percentage of IGT individuals (5-10%) progress to  
20 insulin deficient non-insulin dependent diabetes (NIDDM) each year. Some of these individuals further progress to insulin dependent diabetes mellitus (IDDM). This form of NIDDM or IDDM is associated with decreased release of insulin by pancreatic beta cells and/or a  
25 decreased end-organ response to insulin. Other symptoms of diabetes mellitus and conditions that precede or are associated with diabetes mellitus include: vascular pathologies, peripheral and sensory neuropathies, blindness, and deafness.

30 The nuclear genome has been the main focus of the search for causative genetic mutations for diabetes, AD, PD. However, despite intense effort, nuclear genes that segregate with diabetes, AD, PD are rare, such as mutations in the insulin gene, the insulin receptor  
35 gene, the adenosine deaminase gene and the glucokinase gene.

It has been recognized that some degenerative diseases such as Leber's hereditary optic neuropathy, myoclonus, epilepsy, lactic acidosis and stroke (MELAS), and myoclonic epilepsy ragged red fiber syndrome, are transmitted through mitochondrial DNA mutations. Mitochondrial DNA mutations have also been implicated in explaining the apparently "sporadic" (nonmendelian) occurrence of some degenerative neurologic disorders, such as Parkinson's and Alzheimer's disease. Indeed, most cases of PD appear sporadically in the population; even with identical twins, one may have the disease, and the other not. This suggests that nuclear chromosomal abnormalities are not the cause of this disease. Furthermore, it has been shown that the neurotoxin 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) induces parkinsonism in animals and man. MPTP is converted to its active metabolite, MPP+, in dopamine neurons; it then becomes concentrated in the mitochondria. The MPP+ then selectively inhibits the enzyme NADH:UBIQUINONE OXIDOREDUCTASE ("Complex I"), leading to the increased production of free radicals, reduced production of adenosine triphosphate, and ultimately, the death of affected dopamine neurons.

In addition, the maternal heredity associated with diabetes mellitus suggests that mitochondrial heredity might play a role. Indeed, a rare form of late-onset NIDDM associated with nerve deafness appears to segregate with a point mutation in a mitochondrial tRNA gene (tRNA<sup>leu</sup>). Individuals carrying this mutation often present with impaired insulin secretion in response to glucose and are usually given the diagnosis of insulin dependent diabetes mellitus (IDDM), slowly progressive IDDM, or insulin deficient non-insulin dependent diabetes (NIDDM). Although this mutation accounts for less than 1% of NIDDM cases, it raises the possibility that other mutations in mtDNA may associate with NIDDM.

Proteins encoded by the mitochondrial genome are components of the electron transport chain, and deficits in electron transport function have been reported in Parkinson's and Alzheimer's disease. In particular, it has been reported that defects in cytochrome c oxidase, an important terminal component of the electron transport chain located in the mitochondria of eukaryotic cells, may be involved in Alzheimer's disease.

One report suggesting a relation between AD and cytochrome c oxidase is Parker et al., Neurology 40: 13021303 (1990), which finds that patients with Alzheimer's disease have reduced cytochrome c oxidase activity. The enzyme cytochrome C oxidase (COX), which makes up part of the mitochondrial electron transport chain (ETC), is present in normal amounts in AD patients; however, the catalytic activity of the enzyme in AD patients and in the brains of AD patients at autopsy has been found to be abnormally low. This suggests that the genes for COX in AD patients are defective, leading to decreased catalytic activity that in some fashion causes or contributes to the symptoms that are characteristic of AD. It has also been shown by Bennett et al., J. Geriatric Psychiatry and Neurology 5:93-101 (1992), that when sodium azide, a specific inhibitor of cytochrome c oxidase (COX) was infused into rats, the rats suffered impaired memory and learning (a form of dementia). The rats mimicked the effect of Alzheimer's disease in humans. In addition, the sodium azide-tested rats failed to display long term potentiation, demonstrating loss of neuronal plasticity. It has been hypothesized that the reduced cytochrome c oxidase activity leads to increased intracellular levels of oxygen free radicals, and that the cumulative effects of free radical-mediated lipid oxidation ultimately cause the degenerative neurological changes that are



characteristic of AD. Wallace, D.C., Science, 256:628-632 (1992).

Despite these findings, prior to the present invention, the exact mechanism producing the electron transport dysfunctions was not known for Alzheimer's disease, Parkinson's disease or several forms of diabetes mellitus, including late-onset diabetes. Nor had a genetic or structural basis for these dysfunctions been identified. Without knowing what causes these electron transport dysfunctions and in particular the genetic or structural basis, it is difficult to diagnose these diseases.

Clearly then, a reliable diagnosis of AD, PD, and diabetes mellitus at its earliest stages is critical for efficient and effective intercession and treatment of their debilitating diseases. There is a need for a non-invasive diagnostic assay that is reliable at or before the earliest manifestations of symptoms. There is also a need for developing therapeutic regimens or drugs for treating both the symptoms and the disease itself.

The identification of diagnostic assays and of therapeutic regimens or drugs that are useful in the treatment of disorders associated with mitochondrial defects has historically been hampered by the lack of reliable model systems that could be used in rapid and informative screening. Animal models do not exist for many of the diseases that are associated with mitochondrial gene defects. Appropriate cell culture model systems are either not available, or are difficult to establish and maintain. Furthermore, even when cell culture models are available, it is often not possible to discern whether the mitochondrial or the nuclear genome is responsible for given phenotype, as mitochondrial functions are often encoded by both nuclear and mitochondrial genes. It is, therefore, also not possible to tell whether the apparent effect of a

given drug or treatment operates at the level of the mitochondrial genome or elsewhere.

One approach that has been useful in discerning which genome is responsible is to destroy the  
5 mitochondrial DNA in cultured cells known to have proper mitochondrial function and then transfer to such cells the mitochondria from diseased cells. However, the resulting cell lines, called  $\rho^0$  cell lines, tend to be unstable and hard to culture. Fully differentiated cell  
10 lines are used as the targets for transplantation, but their naturally limited life spans makes them particularly unsuitable for screening purposes. In addition, such transformations have not been done using cells of the type that are most affected by the disease,  
15 making it unclear whether the mitochondrial deficiencies observed in the transformants are related to the disease state being studied. Thus, there is currently a need for reliable model systems that can be used in rapid and informative screening of PD, AD and diabetes mellitus.  
20 The present invention satisfies these needs for a useful diagnostic and effective treatment of PD, AD and diabetes mellitus and provides related advantages, as well.

## 25 SUMMARY OF THE INVENTION

The present invention relates to the identification of genetic mutations in mitochondrial cytochrome c oxidase genes which segregate with a disease state, such as Alzheimer's disease or diabetes mellitus. The  
30 invention provides methods for detecting such mutations as a diagnostic for Alzheimer's disease or diabetes mellitus, either before or after the onset of clinical symptoms.

According to an embodiment of the present invention  
35 for detecting the presence of Alzheimer's disease or diabetes mellitus, a biological sample containing mitochondria from a subject is obtained and one or more

mutations in the sequence of a mitochondrial cytochrome c oxidase gene which correlates with the presence of Alzheimer's disease or diabetes mellitus is interrogated. Such interrogated mutations are preferably positioned between codon 155 and codon 415 of the cytochrome c oxidase I gene and/or between codon 20 and codon 150 of the cytochrome c oxidase II gene. More preferably, the mutations are interrogated at one or more of the following positions: codon 155, codon 167, codon 178, codon 193, codon 194, and codon 415 of the cytochrome c oxidase I gene; and codon 20, codon 22, codon 68, codon 71, codon 74, codon 95, codon 110, and codon 146 of the cytochrome c oxidase II gene. If desired, the codon of interest can be amplified prior to interrogation.

Preferred methods for interrogating the above mutations include: (a) hybridization with oligonucleotide probes, (b) methods based on the ligation of oligonucleotide sequences that anneal adjacent to one another on target nucleic acids, such as the ligase chain reaction, (c) the polymerase chain reaction or variants thereof which depend on using sets of primers, and (d) single nucleotide primer-guided extension assays.

The present invention also encompasses nucleic acid sequences which are useful in the above mentioned diagnostics, namely those which correspond, or are complementary, to portions of mitochondrial cytochrome c oxidase gene that contain gene mutations which correlate with the presence of Alzheimer's disease or diabetes mellitus. According to one embodiment, the nucleic acid sequences are labelled with detectable agents.

Preferred detectable agents include radioisotopes (such as  $^{32}\text{P}$ ), haptens (such as digoxigenin), biotin, enzymes (such as alkaline phosphatase or horseradish peroxidase), fluorophores (such as fluorescein or Texas Red), or chemilumiphores (such as acridine).

According to another embodiment for detecting the presence of Alzheimer's disease or diabetes mellitus, a biological sample is interrogated for the presence of protein products. In particular, protein products of mitochondria with one or more cytochrome c oxidase mutations that correlate with the presence of Alzheimer's disease or diabetes mellitus are interrogated. Preferred agents for the interrogation of such proteins include monoclonal antibodies.

10 According to another embodiment of the present invention, genetic mutations which cause Alzheimer's disease or diabetes mellitus are detected by determining the sequence of mitochondrial cytochrome c oxidase genes from subjects known to have Alzheimer's disease or  
15 diabetes mellitus, and comparing the sequence to that of known wild-type mitochondrial cytochrome c oxidase genes. Other embodiments of the present invention pertain to suppression of the undesired biological activity of the mutations. This affords a therapeutic  
20 treatment for Alzheimer's disease or diabetes mellitus. More specifically, one embodiment of the invention pertains to methods of inhibiting the transcription or translation of mutant cytochrome c oxidase encoding genes by contacting the genes with antisense sequences  
25 which are specific for mutant sequences and which hybridize to a target mutant cytochrome c oxidase gene or messenger RNA transcribed therefrom.

Another embodiment of the invention concerns the selective introduction of a conjugate molecule into  
30 mitochondria with defective cytochrome c oxidase genes. The conjugate comprises a targeting molecule conjugated to a toxin or to an imaging ligand using a linker. The targeting molecule can be, for example, a lipophilic cation such as an acridine orange derivative, a  
35 rhodamine 123 derivative, or a JC-1 (5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidiazolo-carbocyanine iodide) derivative. The linker can

include, for example, an ester, ether, thioether, phosphorodiester, thiophosphorodiester, carbonate, carbamate, hydrazone, oxime, amino or amide functionality. The imaging ligand can be, for example, a radioisotope, hapten, biotin, enzyme, fluorophore or chemilumiphore. And the toxin can be, for example, phosphate, thiophosphate, dinitrophenol, maleimide and antisense oligonucleic acids.

The present invention also provides model systems for diseases that are associated with or caused by defects in mitochondrial metabolism. In addition, it provides methods for the use of these model systems for screening and evaluating drugs and treatments for such disorders. Moreover, it provides methods for using these model systems to diagnose such disorders.

The present invention further provides for the transplantation of mitochondria into undifferentiated germ cells or embryonic cells, thus providing for the maturation of test animals having mitochondria that have been wholly or partially derived from cells of a diseased organism.

By using these same cultures in screening, it is also possible to predict which of several possible drugs or therapies may be best for that particular patient.

The present invention also comprises the transplantation of mitochondria into undifferentiated germ cells or embryonic cells, to yield organisms having mitochondria that have been wholly or partially derived from cells of a diseased organism.

Some embodiments of the present invention offer outstanding opportunities to identify, probe and characterize defective mitochondrial genes and mutations thereof that are associated with diabetes mellitus, to determine their cellular and metabolic phenotypes, and to assess the effects of various drugs and treatment regimens. In one embodiment, mitochondria from cells of a diabetes mellitus patient are transferred to

immortalized  $\beta$  cells. The cells undergo phenotypic changes characteristic of late onset diabetes mellitus; for example, reduced activity of cytochrome C oxidase (COX). If exogenous agents or treatments are used on  
5 such samples and are able to prevent, delay, or attenuate the phenotypic change, then those agents or treatments warrant further study for their ability to prevent, delay or attenuate late onset diabetes mellitus in humans.

10 Because such cell systems are observed to undergo phenotypic changes characteristic of the diseases to which they relate, they also are used as methods of diagnosis. For example, cells are taken from an individual presenting with symptoms of late onset  
15 diabetes mellitus, and the mitochondria from those cells are put into immortalized  $\beta$  cells. Samples of these cultures are then chemically induced to differentiate into cells with pancreatic "beta cell-like" properties (e.g., insulin secretion). If the differentiated cells  
20 that contain the patient's mitochondria begin to exhibit the degenerative phenotype that is characteristic of late onset diabetes mellitus (e.g., decreased insulin secretion), this confirms that the mitochondria carry one or more causative mtDNA mutation. It thus confirms  
25 the diagnosis of late onset diabetes mellitus.

The appended claims are hereby incorporated by reference as a further enumeration of preferred embodiments.

It is an object of the present invention to  
30 identify the structural and genetic basis for the electron transport dysfunctions that are known to accompany mitochondrial disease, such as Alzheimer's disease or diabetes mellitus.

It is another object of the present invention to  
35 provide reliable and efficient means for the diagnosis of Alzheimer's disease or diabetes mellitus.

It is another object of the present invention to provide effective therapies for the treatment of Alzheimer's disease or diabetes mellitus.

It is yet another object of the present invention to provide an immortal  $\rho^0$  cell line.

It is another object of the present invention to provide an immortal  $\rho^0$  cell line that is undifferentiated, but is capable of being induced to differentiate.

It is a further object of the present invention to provide a cybrid cell line, comprising cultured immortal cells having genomic and mitochondrial DNAs of differing biological origins.

It also is an object of the present invention to provide a cybrid cell line, comprising cultured immortal cells having genomic DNA with origins in a neuroblastoma cell line, and mitochondrial DNA having its origin in a human tissue sample derived from an individual with a disorder known to be associated with a mitochondrial defect.

It is also an object of the present invention to provide cell lines whose genomic DNA is derived from cells that maintain a normal pancreatic  $\beta$  cell phenotype (such as, but not limited to,  $\beta$  TC6-F7, HIT, RINm5f, and TC-1 cells) and mitochondrial DNA having its origin in a human tissue sample derived from an individual with a disorder known to be associated with a mitochondrial defect that segregates with late onset diabetes mellitus.

It is further an object of the present invention to provide an immortal  $\rho^0$  cell line that is undifferentiated, but is capable of being induced to differentiate, comprising cultured immortal cells having genomic DNA with origins in immortalized  $\beta$  cells (for example, TC6-F7, HIT-T15, RINm5f, TC-1, and INS-1 cells), and mitochondrial DNA having its origin in a human tissue sample derived from an individual with a

disorder known to be associated with a mitochondrial defect that segregates with late onset diabetes mellitus.

It is also an object of the present invention to  
5 provide model systems for the study of disorders which are associated with mitochondrial defects.

It is another object of the invention to provide model systems for the screening of drugs effective in treating disorders associated with mitochondrial defects  
10 that segregate with late onset diabetes mellitus.

A further object of the present invention is to provide model systems for the evaluation of therapies for effectiveness in treating disorders associated with mitochondrial defects that segregate with late onset  
15 diabetes mellitus.

It is another object of the invention to provide model systems for the screening of drugs effective in treating disorders associated with mitochondrial defects.

20 A further object of the present invention is to provide model systems for the evaluation of therapies for effectiveness in treating disorders associated with mitochondrial defects.

Another object of the invention is to provide model  
25 systems for the diagnosis of disorders associated with mitochondrial defects.

It is a further object to provide methods for the construction of the above-mentioned model systems.

An additional object is to provide methods for  
30 using these model systems for drug screening, therapy evaluation, and diagnosis.

It is a further object of the present invention to provide animal models for diseases that are associated with mitochondrial defects. These animals models are  
35 useful for drug screening, therapy evaluation and diagnosis.



A further object of the present invention is to provide methods of making such animal models.

One advantage of the present invention is that it provides an effective diagnostic of Alzheimer's disease, particularly for the more prevalent form, sporadic AD and diabetes mellitus.

Another advantage of the present invention is that it affords a non-invasive diagnostic that is reliable at or before the earliest manifestations of AD or diabetes mellitus symptoms.

Still another advantage of the present invention is that it provides an effective therapy that addresses the primary cause of AD or diabetes mellitus, by suppressing the undesired biological activity of mutations that segregate with Alzheimer's disease or diabetes mellitus, or by selectively destroying defective mitochondria.

Another advantage offered by the present invention is that it for the first time offers stable cultures of cells that have had their mitochondria transplanted from other cells. Published studies have reported transplanting mitochondria into fully differentiated (mature) cells, but these cells are not maintainable, and eventually the cultures die. In contrast, the present invention teaches that if mitochondria are transplanted into an immortal, differentiatable cell line, the transplanted cells are also immortal. It further teaches the induction of differentiation among a subpopulation of the immortal culture, which allows for the same experiments to be done as would otherwise have been possible had the transplant been made directly into the differentiated cells.

Still another advantage of the present invention is that it offers model systems that have greater relevance to the disorder under study. Published articles used osteosarcoma (bone cancer) cells as the recipients of transplanted mitochondria; however, bone cells are not a primary site of pathogenesis for the neurological

diseases for which those transformants were offered. The present invention contemplates that the immortalized target cells for mitochondrial transplant would be selected such that they would be capable of

5 differentiation into cells of the type that are primarily affected in the disease state under study. For example, in the examples herein, mitochondria from an AD patient are transplanted into neuroblastoma cells, subcultures of which can be induced to differentiate

10 into neurons. The phenotypic expression of the mitochondrial defects in this model system can thus be observed in the very cell type that is most affected by the disease.

Other objects and advantages of the invention and

15 alternative embodiments will readily become apparent to those skilled in the art, particularly after reading the detailed description, and examples set forth below.

#### BRIEF DESCRIPTION OF THE FIGURES

20 Figure 1 lists the 5' end upstream non-coding region, the complete nucleic acid sequence encoding mitochondrial cytochrome c oxidase subunit I and the 3' end downstream non-coding region. (SEQ. ID. NO. 1).

Figure 2 lists the 5' end non-coding region, the

25 complete nucleic acid sequence of the mitochondrial cytochrome c oxidase subunit II coding region and the 3' end downstream non-coding region. (SEQ. ID. NO. 2).

Figure 3 lists the 5' end non-coding region, the complete nucleic acid sequence of the mitochondrial

30 cytochrome c oxidase subunit III coding region and the 3' end downstream non-coding region. (SEQ. ID. NO. 3).

Figure 4 illustrates a reaction scheme for the preparation of several acridine orange derivatives useful for the detection and selective destruction of

35 defective mitochondria.

Figures 5-8 illustrate reaction schemes for the preparation of several JC-1 derivatives useful for the

detection and selective destruction of defective mitochondria.

Figure 9 is a graph showing that cyanide-sensitive oxygen consumption decreases with ethidium bromide treatment, indicating that endogenous mitochondrial oxidative phosphorylation has been disabled;

Figure 10 is a graph showing that ethidium bromide treatment diminishes the sensitivity of cellular oxygen uptake to various electron transport chain inhibitors, confirming that ethidium bromide has disabled the endogenous electron transport chain;

Figure 11 is a graph showing that  $\rho^0$  cells of the present invention are dependent on pyruvate, but not uridine, for growth;

Figure 12 is a graph showing that cells exposed to increasing concentrations of ethidium bromide for 64 days have increasing quantities of inner mitochondrial membrane, indicating that such cells have the large, irregular mitochondria that are characteristic of cells lacking mitochondrial DNA;

Figure 13 is a graph showing that cells treated with ethidium bromide for 64 days and then treated with the cationic dye JC-1 show increased fluorescence, suggesting that the enlarged mitochondria establish increased transmembrane proton gradients even in the absence of mitochondrial DNA.

#### DETAILED DESCRIPTION OF THE INVENTION

The present invention relates to genetic mutations in mitochondrial cytochrome c oxidase genes which segregate with diseases such as diabetes mellitus and Alzheimer's disease. The invention provides methods for detecting such mutations, as a diagnostic for these diseases, either before or after the onset of clinical symptoms. Moreover, the invention also pertains to suppression of the undesired biological activity of the mutations and thus affords a therapeutic treatment for

these diseases. Not only does this invention provide the first effective diagnostic of Alzheimer's disease and diabetes mellitus which is reliable at or before the earliest manifestations of AD or diabetes mellitus  
5 symptoms, it also provides the first effective therapy for these debilitating diseases.

In order to facilitate a full and complete understanding of the present invention, it is important to note that all terms used herein are intended to have  
10 the same meaning as generally ascribed to those terms by those skilled in the art of molecular genetics, unless defined to the contrary. The techniques employed herein are also those that are known to one of ordinary skill in the art, unless stated otherwise. The references  
15 cited herein are incorporated by reference in their entireties.

In using the terms "nucleic acid", RNA, DNA, etc., we do not mean to limit the chemical structures that can be used in particular steps. For example, it is well  
20 known to those skilled in the art that RNA can generally be substituted for DNA, and as such, the use of the term "DNA" should be read by those skilled in the art to include this substitution. In addition, it is known that a variety of nucleic acid analogues and derivatives  
25 can be made and will hybridize to one another and to DNA and RNA, and the use of such analogues and derivatives is also within the scope of the present invention.

The term "gene" includes cDNAs, RNA, or other polynucleotides that encode gene products. The term  
30 "tissue" includes blood and/or cells isolated or suspended from solid body mass, as well as the solid body mass of the various organs. In addition, "expression" of a gene or nucleic acid encompasses not only cellular gene expression, but also the  
35 transcription and translation of nucleic acid(s) in cloning systems and in any other context. "Immortal" cell lines denotes cell lines that are so denoted by

persons of ordinary skill, or are capable of being passaged preferably an indefinite number of times, but not less than ten times, without significant phenotypical alteration. "ρ° cells" are cells  
5 essentially depleted of functional mitochondria and/or mitochondrial DNA, by any method useful for this purpose.

Reference to particular buffers, media, reagents, cells, culture conditions and the like, or to some  
10 subclass of same, is not intended to be limiting, but should be read to include all such related materials that one of ordinary skill in the art would recognize as being of interest or value in the particular context in which that discussion is presented. For example, it is  
15 often possible to substitute one buffer system or culture medium for another, such that a different but known way is used to achieve the same goals as those to which the use of a suggested method, material or composition is directed.

20 Although the cells used in one embodiment herein are neuroblastoma cells, the present invention is not limited to the use of such cells. Cells from different species (human, mouse, etc.) or different tissues (breast epithelium, colon, neuronal tissue, lymphocytes,  
25 etc.) are also useful in the present invention.

#### Segregation of Cytochrome C Oxidase Mutations with Mitochondrial Disease

Cytochrome c oxidase (COX) is an important terminal  
30 component of the electron transport chain located in the mitochondria of eukaryotic cells. Cytochrome c oxidase, also known as complex IV of the electron transport chain, is composed of at least thirteen subunits. At least ten of these subunits are encoded by nuclear  
35 genes; the remaining three subunits (I, II, and III) are encoded by mitochondrial genes. Mitochondrial DNA (mtDNA) is a small circular DNA molecule that is

approximately 17 kB long in humans. The mtDNA encodes for two ribosomal RNAs (rRNA), a complete set of transfer RNAs (tRNA), and thirteen proteins, including three cytochrome c oxidase subunits COX I, COX II, and  
5 COX III.

Most of the mtDNA present in an individual is derived from the mtDNA contained within the ovum at the time of the individual's conception. Mutations in mtDNA sequence which affect all copies of mtDNA in an  
10 individual are known as homoplasmic. Mutations which affect only some copies of mtDNA are known as heteroplasmic and will vary between different mitochondria in the same individual. It should also be noted that most mitochondrially encoded proteins and all  
15 mitochondrially encoded COX proteins are transcribed from the heavy strand of mtDNA. The other strand is called the "light strand," because mtDNA can be separated into heavy and light single strands on the basis of their density.

20 In the present invention, mtDNA from normal individuals, known Alzheimer's patients, and known diabetes mellitus patients are isolated, cloned and sequenced. As expected, a few nondeleterious and apparently random mutations in each gene including some  
25 normal genes, are observed. However, in the AD and diabetes mellitus patients, a small number of homoplasmic or heteroplasmic mutations at common sites are noted. For the three mitochondrial COX subunits, the mutations occurred in one or more of the subunit  
30 clones for each individual. Such mutations are especially observed in the expressed regions of COX subunits I and II of the mtDNA.

According to the present invention, such mutations in COX genes segregate with, and are apparently  
35 sufficient for, Alzheimer's disease and diabetes mellitus. Sporadic AD, which accounts for at least 90% of all AD patients, and diabetes mellitus are segregated

with heteroplasmic mutation(s) in the mtDNA-encoded COX subunits. Detection of these mutations, therefore, is both predictive and diagnostic of Alzheimer's disease and diabetes mellitus.

5        Blood and/or brain samples are harvested and DNA isolated from a number of clinically-classified or autopsy confirmed AD patients, from a number of documented age-matched 'normals' (elderly individuals with no history of AD or any sign of clinical symptoms  
10 of AD) and from age-matched neurodegenerative disease controls (patients with Huntington's disease, parasupranuclear palsy, and so forth). Blood samples were also obtained from a number of diabetes mellitus patients. After cloning of cytochrome c oxidase (COX)  
15 gene fragments, the sequences of multiple clones from each patient are obtained. Compilation of the sequences are made, aligned, and compared with published Cambridge and Genbank sequences (Anderson et al., Nature 290:457-465 (1981)) for known normal human COX genes.  
20 The published Cambridge coding sequences are numbered as follows: COX I is nucleotides 5964 to 7505, COX II is nucleotides 7646 to 8329, and COX III is nucleotides 9267 to 10052. The corresponding sequences are numbered as follows according to Anderson's scheme: COX I is  
25 nucleotides 5904 to 7445, COX II is nucleotides 7586 to 8269, and COX III is nucleotides 9207 to 9992. Id. All reference hereinbelow is made only to the published Cambridge sequences, though it will be appreciated by those of skill in the art that the corresponding  
30 sequences, following a different numbering scheme, including Anderson's, could be used in the invention.

Any variation (mutation, insertion, or deletion) from published sequences is verified by replication and by complementary strand sequencing. Analysis of the  
35 variations in known AD patients indicated a several mutations. Some of the mutations observed are 'silent' mutations resulting in no amino acid changes in the

expressed protein. However, a number of mutations present result in amino acid changes in the corresponding protein. In many instances the corresponding amino acid change may also lead to conformational changes to the COX enzyme.

In cytochrome c oxidase subunit II, for example, the sequence in AD patients varies from the normal sequence in at least one base per gene. The data is summarized in Table 2 hereinbelow. Several of the recurrent mutations observed are believed to result in conformational alterations of the COX enzyme. For example, mutation of the normal ACC observed at codon 22 to ATC results in a change from the normal hydrophilic threonine (Thr) to a hydrophobic isoleucine (Ile). Changes of this type in nucleic acid structure, particularly when occurring in highly conserved areas, are known to disrupt or modify enzymatic activity.

As described more fully hereinbelow, each of the COX genes sequenced shows significant variation from the normal sequence at a number of specific sites, or mutational "hot spots." Moreover, these hot spots generally fall within particular regions of the COX genes. In the first 1,530 bases (510 codons) of COX I, and in particular between codons 155 and 415, codons 155, 167, 178, 193, 194 and 415 have a high degree of mutational similarity in the AD sequences (see Table 1). In COX II, hot spots occur especially in the region between codon 20 and codon 150 and in particular at codons 20, 22, 68, 71, 74, 90, 95, 110 and 146 (see Table 2). In COX III, codons 64, 76, 92, 121, 131, 148, 241 and 247 appear to be highly variable hot spots.

**Mutations observed in COX I gene of Alzheimer's patients**  
Table 1 below is an example of several mutations and the number of times a given mutation is observed in ten clones of mitochondrial cytochrome c oxidase subunit I (COX I) gene for each of 44 Alzheimer's patients. The



mutations listed for the AD patients are relative to the published Cambridge sequences for normal human COX I.

The codon number indicated is determined in a conventional manner from the open reading frame at the

5 5'-end of the gene.

TABLE 1

Codon #	26	52	52	52	66	66	64	88	103	109	111	136	155	155	167	170	178	193	193	193	200	200	216	221	261	276	330	357	369	415	415	416	456	456	468	474	504	
Normal AA	Ala	His	His	His	Ile	Ile	Pro	Gly	Trp	Leu	Leu	Tyr	Val	Val	Thr	Asn	Gln	Val	Val	Val	Leu	Pro	Pro	Asn	Asp	Tyr	Ala	Ser	Val	Asp	Thr	Thr	Ile	Val	Val	Met	Glu	Thr
Normal DNA	GCT	CAC	CAC	CAC	ATC	ATC	CCC	GGT	TGA	CTC	CTC	TAC	GTC	GTC	ACA	AAT	CAA	GTC	GTC	GTC	CTA	CCA	CCA	AAC	GAC	TAC	GCT	AGC	GTA	GAC	ACT	ACT	ATC	GTA	ATA	ATA	ACA	
Observed	Thr	Tyr	Leu	Leu	Thr	Thr	Leu	Asp	Arg	Pro	Pro	His	Ile	Ala	Ala	Ser	Leu	Ala	Ile	Phe	Leu	Ser	Asp	Cys	Thr	Gly	Ala	Gly	Ala	Ile	Thr	Ala	Met	Thr	Val	Gly	Ala	
Mutation	ACT	CCC	CTC	CTC	GTC	ACC	CTC	GAT	CGA	CCC	CCC	CAC	ATC	GCC	GCC	AGT	CTA	GCC	ATC	TTA	CTA	TCA	GAC	AAC	TGC	ACT	GGC	GCA	GGC	GCT	ATT	ACC	GCA	ATA	ACA	GTA	GGA	GCA
AD Patient																																						
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As evidenced by Table 1, mutational hot spots of COX I in AD patients are codons 155, 167, 178, 193, 194 and 415.

5 **Mutations observed in COX II gene of Alzheimer's patients**

Table 2 below is an example of several mutations and the number of times a given mutation is observed in ten clones of mitochondrial cytochrome c oxidase subunit II (COX II) gene for each of the 44 Alzheimer's patients. The mutations listed for the AD patients are relative to the published Cambridge sequences for normal human COX II. The codon number indicated is determined in a conventional manner from the open reading frame at the 5'-end of the gene.

TABLE 2

[illegible]

As evidenced by Table 2, the mutational hot spots of COX II in AD patients are codons 20, 22, 68, 71, 74, 90, 95, 110 and 146.

At each mutational hot spot, the specific variations noted in AD patients appear universally. For example, at codon 415 in COX I, the normal codon is threonine; each of nine AD mutations observed in codon 415 in COX I codes for alanine. At position 194 in COX I, the aromatic phenylalanine codon replaces the normally hydrophobic leucine. These specific mutations do not occur randomly and are not observed in normal or neurological patients which do not have Alzheimer's disease.

Table 3 below demonstrates the use of the above mutational hot spots in the diagnosis of Alzheimer's disease. For each patient in Table 3, the presence of a mutation at each of codons 155, 167, 178, 193, 194 and 415 of COX I, and each of codons 20, 22, 68, 71, 74, 95, 110 and 146 of COX II is indicated by a shaded box.

Blood samples are obtained and DNA isolated from a number of living subjects that are either clinically-classified AD patients ("Blood/AD") or documented age-matched 'normals' (elderly individuals with no family history of AD or any sign of clinical symptoms of AD) ("Blood/Control"). Of the clinically-classified AD patients ("Blood/AD"), 61% (22 out of 36) have mutations at one or more of the above hot spots. 36% (13 out of 36) contain no mutations. However, as noted above, the diagnosis of probable Alzheimer's disease is presently limited to clinical observation, with definitive analysis accomplished only by pathological examination at autopsy. Moreover, of living patients presently diagnosed as having AD by clinical observation only about 70 to 80% are confirmed to have AD upon autopsy. Tierney, M.C. et al., Neurology 38:359-364 (1988). The remaining 20 to 30% are incorrectly diagnosed as having AD, while they actually have another condition such as

senile dementia of the Lewy body variety, Pick's Disease, parasupranuclear palsy, and so forth. Thus, it is expected that a significant percentage of the blood samples taken from living clinically-classified AD patients will not test positive for AD. Indeed, a  
5 contrary result is cause for concern.

Of the living documented age-matched normals (Blood/Control) only 1 out of 14 (7%) had a single hot spot mutation. Moreover, it is noted that this  
10 individual is 65 years old and may yet develop symptoms of AD.

Brain samples are also harvested and DNA isolated from a number of deceased patients that are confirmed to have AD upon pathological examination at autopsy  
15 ("Brain/AD") or deceased documented age matched 'normals' (elderly individuals with no family history of AD, no sign of clinical symptoms of AD during life, and no sign of AD upon pathological examination at autopsy) ("Brain/Control"). Brain samples are also harvested and  
20 DNA isolated from a number of deceased patients that are diagnosed upon autopsy to have other degenerative neurologic disorders selected from Huntington's disease ("Brain/HD"), non-specific degenerative disease ("Brain/NSD"), parasupranuclear palsy ("Brain/PSP"),  
25 Pick's disease (Brain/Picks"), Hallervorden Spatz ("Brain/HSP"), diffuse Lewy body disease ("Brain/DLBD"), atypical tangles ("Brain/AT"), argyrophyllic grains ("Brain/AG"), senile dementia of the Lewy body variety ("Brain/LBV").

30 Results from the DNA isolated from brain samples clearly illustrate the specificity of the diagnostic technique of the present invention. Of the brain samples taken from individuals with pathologically confirmed AD, 83% (10 or 12) contained one or more hot  
35 spot mutations. Of the two remaining individuals (BA and DE), BA demonstrated mutations at COX I codons 170 and 276 and COX II codon 26, while DE demonstrated

mutations at COX I codon 221 and COX II codon 90. Accordingly, it may be desirable to extend to above list of hot spots. In contrast, none of the age matched 'normals' are found to contain such mutations.

5        In addition, of the individuals having other neurologic disorders, only 2 of 18 (11%) contained a single mutation. This illustrates that the diagnosis of the present invention is specific to AD. Moreover, pathologists involved with the autopsy of one of the two  
10 individuals (SC) are unable to definitively clearly differentiate the dementia with argyrophillic grains from AD. Finally, one cannot rule out the possibility that the other individual (KI) would have manifested symptoms of AD if the individual had not succumbed to  
15 Para-Supranuclear Palsy.

TABLE 3

Codon	Wild-type Amino Acid	Wild-type DNA	Observed Amino Acid	Observed DNA	Patient #	Source
155	Val	GTT	Ile	ATC	1KE	Blood/AD
167	Thr	ACA	Ala	GCA	2RI	Blood/AD
178	Gln	CAA	Leu	CTA	3DA	Blood/AD
193	Val	GTC	Ala	GCC	4WO	Blood/AD
194	Leu	CTA	Phe	TTA	5PI	Blood/AD
415	Thr	ACT	Ala	GCT	6TR	Blood/AD
415	Thr	ACT	Ile	ATT	7LF	Blood/AD
71	Ile	ATC	Thr	ACC	8OB	Blood/AD
74	Val	GTC	Ile	ATC	9GE	Blood/AD
95	Leu	CTT	Phe	TTT	10RE	Blood/AD
110	Tyr	TAC	Cys	TGC	11BJ	Blood/AD
110	Tyr	TAC	His	CAC	12ML	Blood/AD
146	Ile	ATT	Val	GTT	13HA	Blood/AD
146	Ile	ATT	Thr	ACT	15AS	Blood/AD
167	Val	GTT	Ile	ATC	16CR	Blood/AD
178	Gln	CAA	Leu	CTA	17CO	Blood/AD
193	Val	GTC	Ala	GCC	18DM	Blood/AD
194	Leu	CTA	Phe	TTA	19JY	Blood/AD
415	Thr	ACT	Ala	GCT	20MI	Blood/AD
415	Thr	ACT	Ile	ATT	21BE	Blood/AD
71	Ile	ATC	Thr	ACC	22SJ	Blood/AD
74	Val	GTC	Ile	ATC	23WY	Blood/AD
95	Leu	CTT	Phe	TTT	24KP	Blood/AD
110	Tyr	TAC	Cys	TGC	25BL	Blood/AD
110	Tyr	TAC	His	CAC	26SD	Blood/AD
146	Ile	ATT	Val	GTT	27HU	Blood/Control
146	Ile	ATT	Thr	ACT	28UT	Blood/Control
167	Val	GTT	Ile	ATC	29OD	Blood/Control



TABLE 3 (cont)

TABLE 3 (cont)

TABLE 3 (cont)

[illegible]

The invention also includes the isolated nucleotide sequences which correspond to or are complementary to portions of mitochondrial cytochrome c oxidase genes which contain gene mutations that correlate with the presence of Alzheimer's disease or diabetes mellitus. The isolated nucleotide sequences which contain gene mutations include COX I nucleotides 5964 to 7505, COX II nucleotides 7646 to 8329 and COX III nucleotides 9267 to 10052.

10

Diagnostic Detection of diseases of Mitochondrial Origin:

According to the present invention, base changes in the mitochondrial COX genes can be detected and used as a diagnostic for diseases of mitochondrial origin, such as Alzheimer's disease and diabetes mellitus. A variety of techniques are available for isolating DNA and RNA and for detecting mutations in the isolated mitochondrial COX genes.

A number of sample preparation methods are available for isolating DNA and RNA from patient blood samples. For example, the DNA from a blood sample is obtained by cell lysis following alkali treatment. Often, there are multiple copies of RNA message per DNA. Accordingly, it is useful from the standpoint of detection sensitivity to have a sample preparation protocol which isolates both forms of nucleic acid. Total nucleic acid may be isolated by guanidium isothiocyanate/phenol-chloroform extraction, or by proteinase K/phenol-chloroform treatment. Commercially available sample preparation methods such as those from Qiagen Inc. (Chatsworth, CA) can also be utilized.

As discussed more fully hereinbelow, mutations can be detected by hybridization with one or more labelled probes containing complements of the mutations. Since mitochondrial diseases can be heteroplasmic (possessing both the mutation and the normal sequence) a

quantitative or semi-quantitative measure (depending on the detection method) of such heteroplasmy can be obtained by comparing the amount of signal from the mutant probe to the amount from the normal or wild-type probe.

A variety of techniques, as discussed more fully hereinbelow, are available for detecting the specific mutations in the mitochondrial COX genes. The detection methods include, for example, cloning and sequencing, ligation of oligonucleotides, use of the polymerase chain reaction and variations thereof, use of single nucleotide primer-guided extension assays, hybridization techniques using target-specific oligonucleotides and sandwich hybridization methods.

Cloning and sequencing of the COX genes can serve to detect mutations in patient samples. Sequencing can be carried out with commercially available automated sequencers utilizing fluorescently labelled primers. An alternate sequencing strategy is the "sequencing by hybridization" method using high density oligonucleotide arrays on silicon chips (Fodor et al., Nature 364:555-556 (1993); Pease et al., Proc. Natl. Acad. Sci. USA, 91:5022-5026 (1994)). For example, fluorescently-labelled target nucleic acid generated, for example from PCR amplification of the target genes using fluorescently labelled primers, are hybridized with a chip containing a set of short oligonucleotides which probe regions of complementarily with the target sequence. The resulting hybridization patterns are useful for reassembling the original target DNA sequence.

Mutational analysis can also be carried out by methods based on ligation of oligonucleotide sequences which anneal immediately adjacent to each other on a target DNA or RNA molecule (Wu and Wallace, Genomics 4:560-569 (1989); Landren et al., Science 241:1077-1080 (1988); Nickerson et al., Proc. Natl. Acad. Sci.

87:8923-8927 (1990); Barany, F., Proc. Natl. Acad. Sci.  
88:189-193 (1991)). Ligase-mediated covalent attachment  
occurs only when the oligonucleotides are correctly  
base-paired. The Ligase Chain Reaction (LCR), which  
5 utilizes the thermostable *Taq* ligase for target  
amplification, is particularly useful for interrogating  
mutation loci. The elevated reaction temperatures  
permits the ligation reaction to be conducted with high  
stringency (Barany, F., PCR Methods and Applications  
10 1:5-16 (1991)).

Analysis of point mutations in DNA can also be  
carried out by using the polymerase chain reaction (PCR)  
and variations thereof. Mismatches can be detected by  
competitive oligonucleotide priming under hybridization  
15 conditions where binding of the perfectly matched primer  
is favored (Gibbs et al., Nucl. Acids. Res. 17:2437-2448  
(1989)). In the amplification refractory mutation  
system technique (ARMS), primers are designed to have  
perfect matches or mismatches with target sequences  
20 either internal or at the 3' residue (Newton et al.,  
Nucl. Acids. Res. 17:2503-2516 (1989)). Under  
appropriate conditions, only the perfectly annealed  
oligonucleotide functions as a primer for the PCR  
reaction, thus providing a method of discrimination  
25 between normal and mutant sequences.

Genotyping analysis of the COX genes can also be  
carried out using single nucleotide primer-guided  
extension assays, where the specific incorporation of  
the correct base is provided by the high fidelity of the  
30 DNA polymerase (Syvanen et al., Genomics 8:684-692  
(1990); Kuppuswamy et al., Proc. Natl. Acad. Sci. U.S.A.  
88:1143-1147 (1991)). Another primer extension assay,  
which allows for the quantification of heteroplasmy by  
simultaneously interrogating both wild-type and mutant  
35 nucleotides, is disclosed in co-pending U.S. patent  
application Serial No. \_\_\_\_\_, filed on March 24, 1995,  
entitled "Multiplexed Primer Extension Methods" and

naming Eoin Fahy and Soumitra Ghosh as inventors, the disclosure of which is hereby incorporated by reference.

Detection of single base mutations in target nucleic acids can be conveniently accomplished by

5 differential hybridization techniques using target-specific oligonucleotides (Suggs et al., Proc. Natl. Acad. Sci. 78:6613-6617 (1981); Conner et al., Proc. Natl. Acad. Sci. 80:278-282 (1983); Saiki et al., Proc. Natl. Acad. Sci. 86:6230-6234 (1989)). For

10 example, mutations are diagnosed on the basis of the higher thermal stability of the perfectly matched probes as compared to the mismatched probes. The hybridization reactions may be carried out in a filter-based format, in which the target nucleic acids are immobilized on

15 nitrocellulose or nylon membranes and probed with oligonucleotide probes. Any of the known hybridization formats may be used, including Southern blots, slot blots, "reverse" dot blots, solution hybridization, solid support based sandwich hybridization, bead-based,

20 silicon chip-based and microtiter well-based hybridization formats.

An alternative strategy involves detection of the COX genes by sandwich hybridization methods. In this strategy, the mutant and wild-type (normal) target

25 nucleic acids are separated from non-homologous DNA/RNA using a common capture oligonucleotide immobilized on a solid support and detected by specific oligonucleotide probes tagged with reporter labels. The capture oligonucleotides can be immobilized on microtitre plate

30 wells or on beads (Gingeras et al., J. Infect. Dis. 164:1066-1074 (1991); Richman et al., Proc. Natl. Acad. Sci. 88:11241-11245 (1991)).

While radio-isotopic labeled detection oligonucleotide probes are highly sensitive,

35 non-isotopic labels are preferred due to concerns about handling and disposal of radioactivity. A number of strategies are available for detecting target nucleic

acids by non-isotopic means (Matthews et al., Anal. Biochem., 169:1-25 (1988)). The non-isotopic detection method may be direct or indirect.

The indirect detection process is generally where  
5 the oligonucleotide probe is covalently labelled with a hapten or ligand such as digoxigenin (DIG) or biotin. Following the hybridization step, the target-probe duplex is detected by an antibody- or streptavidin-enzyme complex. Enzymes commonly used in  
10 DNA diagnostics are horseradish peroxidase and alkaline phosphatase. One particular indirect method, the Genius™ detection system (Boehringer Mannheim) is especially useful for mutational analysis of the mitochondrial COX genes. This indirect method uses  
15 digoxigenin as the tag for the oligonucleotide probe and is detected by an anti-digoxigenin-antibody-alkaline phosphatase conjugate.

Direct detection methods include the use of fluorophor-labeled oligonucleotides, lanthanide  
20 chelate-labeled oligonucleotides or oligonucleotide-enzyme conjugates. Examples of fluorophor labels are fluorescein, rhodamine and phthalocyanine dyes. Examples of lanthanide chelates include complexes of  $\text{Eu}^{3+}$  and  $\text{Tb}^{3+}$ . Directly labeled  
25 oligonucleotide-enzyme conjugates are preferred for detecting point mutations when using target-specific oligonucleotides as they provide very high sensitivities of detection.

Oligonucleotide-enzyme conjugates can be prepared  
30 by a number of methods (Jablonski et al., Nucl. Acids Res., 14:6115-6128 (1986); Li et al., Nucl. Acids Res. 15:5275-5287 (1987); Ghosh et al., Bioconjugate Chem. 1: 71-76 (1990)), and alkaline phosphatase is the enzyme of choice for obtaining high sensitivities of detection.  
35 The detection of target nucleic acids using these conjugates can be carried out by filter hybridization



methods or by bead-based sandwich hybridization (Ishii et al., Bioconjugate Chemistry 4:34-41 (1993)).

Detection of the probe label may be accomplished by the following approaches. For radioisotopes, detection is by autoradiography, scintillation counting or phosphor imaging. For hapten or biotin labels, detection is with antibody or streptavidin bound to a reporter enzyme such as horseradish peroxidase or alkaline phosphatase, which is then detected by enzymatic means. For fluorophor or lanthanide-chelate labels, fluorescent signals may be measured with spectrofluorimeters with or without time-resolved mode or using automated microtitre plate readers. With enzyme labels, detection is by color or dye deposition (p-nitrophenyl phosphate or 5-bromo-4-chloro-3-indolyl phosphate/nitroblue tetrazolium for alkaline phosphatase and 3,3'-diaminobenzidine-NiCl<sub>2</sub> for horseradish peroxidase), fluorescence (e.g. 4-methyl umbelliferyl phosphate for alkaline phosphatase) or chemiluminescence (the alkaline phosphatase dioxetane substrates LumiPhos 530 from Lumigen Inc., Detroit MI or AMPPD and CSPD from Tropix, Inc.). Chemiluminescent detection may be carried out with X-ray or polaroid film or by using single photon counting luminometers. This is the preferred detection format for alkaline phosphatase labelled probes.

The oligonucleotide probes for detection preferably range in size between 10 and 100 bases, more preferably between 15 and 30 bases in length. Examples of such nucleotide probes are found below in Tables 4 and 5. Tables 4 and 5 provide representative sequences of probes for detecting AD mutations in COX genes and representative antisense sequences. In order to obtain the required target discrimination using the detection oligonucleotide probes, the hybridization reactions are preferably run between 20°C and 60°C, and more preferably between 30°C and 55°C. As known to those

skilled in the art, optimal discrimination between perfect and mismatched duplexes can be obtained by manipulating the temperature and/or salt concentrations or inclusion of formamide in the stringency washes.

TABLE 4 Sense Probes -- DNA detection of antisense strand

GENE	AA NO.	LENGTH (WT)	% GC	WILD-TYPE	SEQ. ID. NO.	MUTANT	SEQ. ID. NO.
COXI	155	23	52.2	5'-ACCTAGCAGGTGCTCTCTATG-3'	4	5'-ACCTAGCAGGTATCTCTCTATCT-3'	18
COXI	167	27	22.2	5'-CAATTTTCATCACAACAATATCAATAT-3'	5	5'-CAATTTTCATCAGCAATATATCAATAT-3'	19
COXI	178	21	47.6	5'-GCCATACCCGATACCAACG-3'	6	5'-GCCATACCCGATACCAACG-3'	20
COXI	193	23	47.8	5'-AATCAGCAGGTCTTCTCTCTCC-3'	7	5'-AATCAGCAGGTCTTCTCTCTCC-3'	21
						5'-AATCAGCAGGTCTTCTCTCTCC-3'	22
COXI	194	25	50.0	5'-TCACAGCAGTCTTCTCTCTATC-3'	8	5'-TCACAGCAGTCTTCTCTCTATC-3'	23
COXI	415	26	26.9	5'-CAAAATCCATTTCACTATCATATTC-3'	9	5'-AAATCCATTTTCGCTATCATATTC-3'	24
COXII	20	25	37.5	5'-TCATAGAGAGCTTATCACCCTTTCA-3'	10	5'-TCATAGAGAGCTTATCACCCTTTCA-3'	25
COXII	22	24	37.5	5'-AGAGCTTATCACCCTTTTCATGATCA-3'	11	5'-AGAGCTTATCATCTTTTCATGATCA-3'	26
COXII	68	18	61.1	5'-TGCCCGCCATCATCTAG-3'	12	5'-TGAATATCTTGCCCGCC-3'	27
COXII	71	18	61.1	5'-TGCCCGCAICATCTAG-3'	13	5'-TGCCCGCACCCTCTAG-3'	28
COXII	74	21	52.4	5'-ATCATCTAGTCTCATCGCC-3'	14	5'-ATCATCTTAATCTCATCGCC-3'	29
COXII	95	21	47.6	5'-GATCCCTCCCTTACCATCAA-3'	15	5'-GATCCCTCCCTTACCATCAAT-3'	30
						5'-GATCCCTCCCTTACCATCAA-3'	31
COXII	110	23	52.2	5'-AACCLACGAGACCGACTAG-3'	16	5'-AACCTACGAGCAGCGACTAG-3'	32
						5'-AACCTACGAGTGCAGCGACTAG-3'	33
COXII	146	20	55.0	5'-AGTACTCCCGATTGAAGCC-3'	17	5'-AGTACCCGGTTGAAGCC-3'	34

TABLE 5 Antisense Probes -- DNA and RNA detection of sense sequence

GENE	AA NO.	LENGTH (WT)	% GC	WILD-TYPE	SEQ. ID. NO.	MUTANT	SEQ. ID. NO.
COXI	155	23	52.2	5'-GATAGAGGAGCACCTGCTAGGT-3'	35	5'-AGATAGAGGAGATACCTGCTAGGT-3'	49
COXI	167	27	22.2	5'-ATATTGATAATTGTTGATGATGAATTG-3'	36	5'-ATATTGATAATTGCTGIGATGAATTG-3'	50
COXI	178	21	47.6	5'-CGTTTGGTATGGTTATGGC-3'	37	5'-CGTTTGGTATAGGGTTATGGC-3'	51
COXI	193	23	47.8	5'-GGAGAAGTAGGCTGCTGTAAT-3'	38	5'-GGAGAAGTAGGCTGCTGTAAT-3'	52
COXI	194	25	50.0	5'-GATAGGAGAGTAGGACTGCTGTGA-3'	39	5'-GATAGGAGAGTAGGACTGCTGTGA-3'	53
COXI	415	26	26.9	5'-TGAATATGATAGTGAATGGATTTTG-3'	40	5'-TGAATATGATAGCGAATGGATTTT-3'	55
COXII	20	25	37.5	5'-TGAAGGTGATAGCTCTTCTATGA-3'	41	5'-TGAAGGTGATAGGCTCTTCTATGA-3'	56
COXII	22	24	37.5	5'-TGATCATGAAGGTGATAGCTCTT-3'	42	5'-TGATCATGAAGATGATAGCTCT-3'	57
COXII	68	18	61.1	5'-GGCGGCGAGTAGTTCA-3'	43	5'-GGCGGCGCAGATAGTTCA-3'	58
COXII	71	18	61.1	5'-CTAGGATGATGCGGCA-3'	44	5'-GGCGGCGCAGATAGTTCA-3'	59
COXII	74	21	52.4	5'-GGCGATGCCACTAGGATGAT-3'	45	5'-GGCGATGAGGATTAGGATGAT-3'	60
COXII	95	21	47.6	5'-TTTGATGTAAAGGGAGGATC-3'	46	5'-ATTGATGTAAAGGGAGGATC-3'	61
COXII	110	23	52.2	5'-CGTAGTCGGTGTACTGCTAGGTT-3'	47	5'-GTAGTCGGTCTCTCTAGGTT-3'	63
COXII	110	23	52.2			5'-GTAGTCGGTGCACCTCTAGGTT-3'	64
COXII	146	20	55.0	5'-GGGCTTCATATCGGAGTACT-3'	48	5'-GGGCTCAACCGGAGTACT-3'	65

As an alternative to detection of mutations in the nucleic acids associated with the COX genes, it is also possible to analyze the protein products of the COX genes. In particular, point mutations in cytochrome c oxidase subunits 1 and 2 are expected to alter the structure of the proteins for which these gene encode. These altered proteins (variant polypeptides) can be isolated and used to prepare antisera and monoclonal antibodies that specifically detect the products of the mutated genes and not those of non-mutated or wild-type genes. Mutated gene products also can be used to immunize animals for the production of polyclonal antibodies. Recombinantly produced peptides can also be used to generate polyclonal antibodies. These peptides may represent small fragments of gene products produced by expressing regions of the mitochondrial genome containing point mutations.

More particularly, as discussed, for example, in PCT/US93/10072, variant polypeptides from point mutations in cytochrome c oxidase subunits 1 and 2 can be used to immunize an animal for the production of polyclonal antiserum. For example, a recombinantly produced fragment of a variant polypeptide can be injected into a mouse along with an adjuvant so as to generate an immune response. Murine immunoglobulins which bind the recombinant fragment with a binding affinity of at least  $1 \times 10^7 \text{ M}^{-1}$  can be harvested from the immunized mouse as an antiserum, and may be further purified by affinity chromatography or other means. Additionally, spleen cells are harvested from the mouse and fused to myeloma cells to produce a bank of antibody-secreting hybridoma cells. The bank of hybridomas can be screened for clones that secrete immunoglobulins which bind the recombinantly produced fragment with an affinity of at least  $1 \times 10^6 \text{ M}^{-1}$ . More specifically, immunoglobulins that selectively bind to the variant polypeptides but poorly or not at all to

wild-type polypeptides are selected, either by pre-absorption with wild-type proteins or by screening of hybridoma cell lines for specific idiotypes that bind the variant, but not wild-type, polypeptides.

5 Nucleic acid sequences capable of ultimately expressing the desired variant polypeptides can be formed from a variety of different polynucleotides (genomic or cDNA, RNA, synthetic oligonucleotides, etc.) as well as by a variety of different techniques.

10 The DNA sequences can be expressed in hosts after the sequences have been operably linked to (i.e., positioned to ensure the functioning of) an expression control sequence. These expression vectors are typically replicable in the host organisms either as  
15 episomes or as an integral part of the host chromosomal DNA. Commonly, expression vectors can contain selection markers (e.g., markers based on tetracycline resistance or hygromycin resistance) to permit detection and/or selection of those cells transformed with the desired  
20 DNA sequences. Further details can be found in U.S. Patent No. 4,704,362.

Polynucleotides encoding a variant polypeptide may include sequences that facilitate transcription (expression sequences) and translation of the coding  
25 sequences such that the encoded polypeptide product is produced. Construction of such polynucleotides is well known in the art. For example, such polynucleotides can include a promoter, a transcription termination site (polyadenylation site in eukaryotic expression hosts), a  
30 ribosome binding site, and, optionally, an enhancer for use in eukaryotic expression hosts, and, optionally, sequences necessary for replication of a vector.

*E. coli* is one prokaryotic host useful particularly for cloning DNA sequences of the present invention.  
35 Other microbial hosts suitable for use include bacilli, such as *Bacillus subtilis*, and other enterobacteriaceae, such as *Salmonella*, *Serratia*, and various *Pseudomonas*

species. In these prokaryotic hosts one can also make expression vectors, which will typically contain expression control sequences compatible with the host cell (e.g. an origin of replication). In addition, any  
5 number of a variety of well-known promoters will be present, such as the lactose promoter system, a tryptophan (Trp) promoter system, a beta-lactamase promoter system, or a promoter system from phage lambda. The promoters will typically control expression,  
10 optionally with an operator sequence, and have ribosome binding site sequences, for example, for initiating and completing transcription and translation.

Other microbes, such as yeast, may also be used for expression. *Saccharomyces* can be a suitable host, with  
15 suitable vectors having expression control sequences, such as promoters, including 3-phosphoglycerate kinase or other glycolytic enzymes, and an origin of replication, termination sequences, etc. as desired.

In addition to microorganisms, mammalian tissue  
20 cell culture may also be used to express and produce the polypeptides of the present invention. Eukaryotic cells are actually preferred, because a number of suitable host cell lines capable of secreting intact human proteins have been developed in the art, and include the  
25 CHO cell lines, various COS cell lines, HeLa cells, myeloma cell lines, Jurkat cells, and so forth. Expression vectors for these cells can include expression control sequences, such as an origin of replication, a promoter, an enhancer, and necessary  
30 information processing sites, such as ribosome binding sites, RNA splice sites, polyadenylation sites, and transcriptional terminator sequences. Preferred expression control sequences are promoters derived from immunoglobulin genes, SV40, Adenovirus, Bovine Papilloma  
35 Virus, and so forth. The vectors containing the DNA segments of interest (e.g., polypeptides encoding a variant polypeptide) can be transferred into the host

cell by well-known methods, which vary depending on the type of cellular host. For example, calcium chloride transfection is commonly utilized for prokaryotic cells, whereas calcium phosphate treatment or electroporation  
5 may be used for other cellular hosts.

The method lends itself readily to the formulation of test kits which can be utilized in diagnosis. Such a kit would comprise a carrier compartmentalized to receive in close confinement one or more containers  
10 wherein a first container may contain suitably labeled DNA probes. Other containers may contain reagents useful in the localization of the labeled probes, such as enzyme substrates. Still other containers may contain restriction enzymes, buffers etc., together with  
15 instructions for use.

Therapeutic treatment of Diseases of Mitochondrial Origin:

Suppressing the effects of the mutations through  
20 antisense technology provides an effective therapy for diseases of mitochondrial origin, such as AD and diabetes mellitus. Much is known about 'antisense' therapies targeting messenger RNA (mRNA) or nuclear DNA. Hélenè et al., Biochem. Biophys. Acta 1049:99-125  
25 (1990). The diagnostic test of the present invention is useful for determining which of the specific AD or diabetes mellitus mutations exist in a particular patient; this allows for "custom" treatment of the patient with antisense oligonucleotides only for the  
30 detected mutations. This patient-specific antisense therapy is also novel, and minimizes the exposure of the patient to any unnecessary antisense therapeutic treatment. As used herein, an "antisense" oligonucleotide is one that base pairs with single  
35 stranded DNA or RNA by Watson-Crick base pairing and with duplex target DNA via Hoogsteen hydrogen bonds.



Without wishing to be held to any particular theory, it has been postulated that the destructive effects of mutations in the cytochrome c oxidase gene arise from the production of the radicals due to faults in the electron transport chain. The effects of such free radicals is expected to be cumulative, especially in view of the lack of mechanisms for suppressing mutations in mitochondria.

The destructive effect of the AD and diabetes mellitus mutations in cytochrome c oxidase genes is preferably reduced or eliminated using antisense oligonucleotide agents. Such antisense agents target mitochondrial DNA, by triplex formation with double-stranded DNA, by duplex formation with single-stranded DNA during transcription, or both. In a preferred embodiment, antisense agents target messenger RNA coding for the mutated cytochrome c oxidase gene(s). Since the sequences of both the DNA and the mRNA are the same, it is not necessary to determine accurately the precise target to account for the desired effect. Procedures for inhibiting gene expression in cell culture and *in vivo* can be found, for example, in C.F. Bennett, et al. J. Liposome Res., 3:85 (1993) and C. Wahlestedt, et al. Nature, 363:260 (1993).

Antisense oligonucleotide therapeutic agents demonstrate a high degree of pharmaceutical specificity. This allows the combination of two or more antisense therapeutics at the same time, without increased cytotoxic effects. Thus, when a patient is diagnosed as having two or more mutations in COX genes, the therapy is preferably tailored to treat the multiple mutations simultaneously. When combined with the present diagnostic test, this approach to "patient-specific therapy" results in treatment restricted to the specific mutations detected in a patient. This patient-specific therapy circumvents the need for 'broad spectrum' antisense treatment using all possible mutations. The

end result is less costly treatment, with less chance for toxic side effects.

One method to inhibit the synthesis of proteins is through the use of antisense or triplex  
5 oligonucleotides, analogues or expression constructs. These methods entail introducing into the cell a nucleic acid sufficiently complementary in sequence so as to specifically hybridize to the target gene or to mRNA. In the event that the gene is targeted, these methods  
10 can be extremely efficient since only a few copies per cell are required to achieve complete inhibition. Antisense methodology inhibits the normal processing, translation or half-life of the target message. Such methods are well known to one skilled in the art.

15 Antisense and triplex methods generally involve the treatment of cells or tissues with a relatively short oligonucleotide, although longer sequences can be used to achieve inhibition. The oligonucleotide can be either deoxyribo- or ribonucleic acid and must be of  
20 sufficient length to form a stable duplex or triplex with the target RNA or DNA at physiological temperatures and salt concentrations. It should also be sufficiently complementary or sequence specific to specifically hybridize to the target nucleic acid. Oligonucleotide  
25 lengths sufficient to achieve this specificity are preferably about 10 to 60 nucleotides long, more preferably about 10 to 20 nucleotides long. However, hybridization specificity is not only influenced by length and physiological conditions but may also be  
30 influenced by such factors as GC content and the primary sequence of the oligonucleotide. Such principles are well known in the art and can be routinely determined by one who is skilled in the art.

As an example, many of the oligonucleotide  
35 sequences used in connection with probes in Tables 4 and 5 can also be used as antisense agents for AD, directed

to either the mitochondrial DNA or resultant messenger RNA.

A great range of antisense sequences can be designed for a given mutation. For example,  
 5 oligonucleotide sequences can be selected from the following list to function as RNA and DNA antisense sequences for the mutant mitochondrial gene COX1, Codon 193.

As can be seen, permutations can be generated for a  
 10 selected mutant antigen by truncating the 5' end, truncating the 3' end, extending the 5' end, or extending the 3' end. Both light chain and heavy chain mtDNA can be targeted. Other variations such as truncating the 5' end and truncating the 3' end,  
 15 extending the 5' end and extending the 3' end, and truncating the 5' end and extending the 3' end, extending the 5' end and truncating the 3' end, and so forth are possible.

20 Antigene to heavy chain mtDNA, wild-type sequence:

SEQ ID NO: 7            5'-AAT CAC AGC AGT CCT ACT TCT CC

Antigene to heavy chain mtDNA, mutant sequence:

SEQ ID NO: 21           5'-AAT CAC AGC AGC CCT ACT TCT CC

25

3' truncation:

SEQ ID NO: 66           5'-AAT CAC AGC AGC CCT ACT TCT C

SEQ ID NO: 67           5'-AAT CAC AGC AGC CCT ACT TCT

SEQ ID NO: 68           5'-AAT CAC AGC AGC CCT ACT TC

30 SEQ ID NO: 69           5'-AAT CAC AGC AGC CCT ACT T

SEQ ID NO: 70           5'-AAT CAC AGC AGC CCT ACT

SEQ ID NO: 71           5'-AAT CAC AGC AGC CCT AC

SEQ ID NO: 72           5'-AAT CAC AGC AGC CCT A

35

5' truncation:

SEQ ID NO: 73           5'-AT CAC AGC AGC CCT ACT TCT CC

SEQ ID NO: 74           5'-T CAC AGC AGC CCT ACT TCT CC

SEQ ID NO: 75           5'-CAC AGC AGC CCT ACT TCT CC

SEQ ID NO: 76           5'-AC AGC AGC CCT ACT TCT CC

40 SEQ ID NO: 77           5'-C AGC AGC CCT ACT TCT CC

SEQ ID NO: 78           5'-AGC AGC CCT ACT TCT CC

**3' and 5' truncation:**

SEQ ID NO: 79 5'-AT CAC AGC AGC CCT ACT TCT C  
 SEQ ID NO: 80 5'-T CAC AGC AGC CCT ACT TCT  
 SEQ ID NO: 81 5'-CAC AGC AGC CCT ACT TC  
 5 SEQ ID NO: 82 5'-AC AGC AGC CCT ACT T

**5' and 3' extension**

SEQ ID NO: 83 5'-C CGT CCT AAT CAC AGC AGC CCT ACT TCT CCT ATC TCT  
 SEQ ID NO: 84 5'-CGT CCT AAT CAC AGC AGC CCT ACT TCT CCT ATC TCT  
 10 SEQ ID NO: 85 5'-GT CCT AAT CAC AGC AGC CCT ACT TCT CCT ATC TCT  
 SEQ ID NO: 86 5'-T CCT AAT CAC AGC AGC CCT ACT TCT CCT ATC TCT  
 SEQ ID NO: 87 5'-CCT AAT CAC AGC AGC CCT ACT TCT CCT ATC TCT  
 SEQ ID NO: 88 5'-CT AAT CAC AGC AGC CCT ACT TCT CCT ATC TCT  
 SEQ ID NO: 89 5'-T AAT CAC AGC AGC CCT ACT TCT CCT ATC TCT

15

**5' extension, 3' extension, or both, keeping length constant:**

SEQ ID NO: 90 5'-C CGT CCT AAT CAC AGC AGC CCT ACT TCT CC  
 SEQ ID NO: 91 5'-CGT CCT AAT CAC AGC AGC CCT ACT TCT CCT  
 SEQ ID NO: 92 5'-GT CCT AAT CAC AGC AGC CCT ACT TCT CCT A  
 20 SEQ ID NO: 93 5'-T CCT AAT CAC AGC AGC CCT ACT TCT CCT AT  
 SEQ ID NO: 94 5'-CCT AAT CAC AGC AGC CCT ACT TCT CCT ATC  
 SEQ ID NO: 95 5'-CT AAT CAC AGC AGC CCT ACT TCT CCT ATC T  
 SEQ ID NO: 96 5'-T AAT CAC AGC AGC CCT ACT TCT CCT ATC TC  
 SEQ ID NO: 97 5'-AAT CAC AGC AGC CCT ACT TCT CCT ATC TCT

25

**Antigene to light chain mtDNA, wild-type sequence:**

SEQ ID NO: 98 3'-TTA GTG TCG TCA GGA TGA AGA GG

**Antigene to light chain mtDNA, mutant sequence:**

30 SEQ ID NO: 99 3'-TTA GTG TCG TCC GGA TGA AGA GG

**5' truncation:**

SEQ ID NO: 100 3'-TTA GTG TCG TCC GGA TGA AGA G  
 SEQ ID NO: 101 3'-TTA GTG TCG TCC GGA TGA AGA  
 35 SEQ ID NO: 102 3'-TTA GTG TCG TCC GGA TGA AG  
 SEQ ID NO: 103 3'-TTA GTG TCG TCC GGA TGA A  
 SEQ ID NO: 104 3'-TTA GTG TCG TCC GGA TGA  
 SEQ ID NO: 105 3'-TTA GTG TCG TCC GGA TG  
 SEQ ID NO: 106 3'-TTA GTG TCG TCC GGA T

40

**3' truncation:**

SEQ ID NO: 107 3'-TA GTG TCG TCC GGA TGA AGA GG  
 SEQ ID NO: 108 3'-A GTG TCG TCC GGA TGA AGA GG  
 SEQ ID NO: 109 3'-GTG TCG TCC GGA TGA AGA GG  
 45 SEQ ID NO: 110 3'-TG TCG TCC GGA TGA AGA GG  
 SEQ ID NO: 111 3'-G TCG TCC GGA TGA AGA GG  
 SEQ ID NO: 112 3'-TCG TCC GGA TGA AGA GG

**3' and 5' truncation:**

50 SEQ ID NO: 113 3'-TA GTG TCG TCC GGA TGA AGA G

SEQ ID NO: 114      3'-A GTG TCG TCC GGA TGA AGA  
 SEQ ID NO: 115      3'-GTG TCG TCC GGA TGA AG  
 SEQ ID NO: 116      3'-TG TCG TCC GGA TGA A

5                    3' and 5' extension:

SEQ ID NO: 117      3'-G GCA GGA TTA GTG TCG TCC GGA TGA AGA GGA TAG AGA  
 SEQ ID NO: 118      3'-GCA GGA TTA GTG TCG TCC GGA TGA AGA GGA TAG AGA  
 SEQ ID NO: 119      3'-CA GGA TTA GTG TCG TCC GGA TGA AGA GGA TAG AGA  
 SEQ ID NO: 120      3'-A GGA TTA GTG TCG TCC GGA TGA AGA GGA TAG AGA  
 10 SEQ ID NO: 121      3'-GGA TTA GTG TCG TCC GGA TGA AGA GGA TAG AGA  
 SEQ ID NO: 122      3'-GA TTA GTG TCG TCC GGA TGA AGA GGA TAG AGA  
 SEQ ID NO: 123      3'-A TTA GTG TCG TCC GGA TGA AGA GGA TAG AGA

5' extension, 3' extension, or both, keeping length constant:

15 SEQ ID NO: 124      3'-G GCA GGA TTA GTG TCG TCC GGA TGA AGA GG  
 SEQ ID NO: 125      3'-GCA GGA TTA GTG TCG TCC GGA TGA AGA GGA  
 SEQ ID NO: 126      3'-CA GGA TTA GTG TCG TCC GGA TGA AGA GGA T  
 SEQ ID NO: 127      3'-A GGA TTA GTG TCG TCC GGA TGA AGA GGA TA  
 SEQ ID NO: 128      3'-GGA TTA GTG TCG TCC GGA TGA AGA GGA TAG  
 20 SEQ ID NO: 129      3'-GA TTA GTG TCG TCC GGA TGA AGA GGA TAG A  
 SEQ ID NO: 130      3'-A TTA GTG TCG TCC GGA TGA AGA GGA TAG AG  
 SEQ ID NO: 131      3'-TTA GTG TCG TCC GGA TGA AGA GGA TAG AGA

The composition of the antisense or triplex  
 25 oligonucleotides can also influence the efficiency of  
 inhibition. For example, it is preferable to use  
 oligonucleotides that are resistant to degradation by  
 the action of endogenous nucleases. Nuclease resistance  
 will confer a longer in vivo half-life to the  
 30 oligonucleotide thus increasing its efficacy and  
 reducing the required dose. Greater efficacy may also  
 be obtained by modifying the oligonucleotide so that it  
 is more permeable to cell membranes. Such modifications  
 are well known in the art and include the alteration of  
 35 the negatively charged phosphate backbone bases, or  
 modification of the sequences at the 5' or 3' terminus  
 with agents such as intercalators and crosslinking  
 molecules. Specific examples of such modifications  
 include oligonucleotide analogs that contain  
 40 methylphosphonate (Miller, P.S., Biotechnology,  
 2:358-362 (1991)), phosphorothioate (Stein, Science  
 261:1004-1011 (1993)) and phosphorodithioate linkages  
 (Brill, W. K-D., J. Am. Chem. Soc., 111:2322 (1989)).

Other types of linkages and modifications exist as well, such as a polyamide backbone in peptide nucleic acids (Nielson et al., Science 254:1497 (1991)), formacetal (Matteucci, M., Tetrahedron Lett. 31:2385-2388 (1990))  
5 carbamate and morpholine linkages as well as others known to those skilled in the art. In addition to the specificity afforded by the antisense agents, the target RNA or genes can be irreversibly modified by  
10 incorporating reactive functional groups in these molecules which covalently link the target sequences e.g. by alkylation.

Recombinant methods known in the art can also be used to achieve the antisense or triplex inhibition of a target nucleic acid. For example, vectors containing  
15 antisense nucleic acids can be employed to express protein or antisense message to reduce the expression of the target nucleic acid and therefore its activity. Such vectors are known or can be constructed by those skilled in the art and should contain all expression  
20 elements necessary to achieve the desired transcription of the antisense or triplex sequences. Other beneficial characteristics can also be contained within the vectors such as mechanisms for recovery of the nucleic acids in a different form. Phagemids are a specific example of  
25 such beneficial vectors because they can be used either as plasmids or as bacteriophage vectors. Examples of other vectors include viruses, such as bacteriophages, baculoviruses and retroviruses, cosmids, plasmids, liposomes and other recombination vectors. The vectors  
30 can also contain elements for use in either procaryotic or eukaryotic host systems. One of ordinary skill in the art will know which host systems are compatible with a particular vector.

The vectors can be introduced into cells or tissues  
35 by any one of a variety of known methods within the art. Such methods are described for example in Sambrook et al., Molecular Cloning: A Laboratory Manual, Cold Spring

Harbor Laboratory, New York (1992), which is hereby incorporated by reference, and in Ausubel et al., Current Protocols in Molecular Biology, John Wiley and Sons, Baltimore, MD (1989), which is also hereby  
5 incorporated by reference. The methods include, for example, stable or transient transfection, lipofection, electroporation and infection with recombinant viral vectors. Introduction of nucleic acids by infection offers several advantages over the other listed methods  
10 which includes their use in both in vitro and in vivo settings. Higher efficiency can also be obtained due to their infectious nature. Moreover, viruses are very specialized and typically infect and propagate in specific cell types. Thus, their natural specificity  
15 can be used to target the antisense vectors to specific cell types in vivo or within a tissue or mixed culture of cells. Viral vectors can also be modified with specific receptors or ligands to alter target specificity through receptor mediated events.

20 A specific example of a viral vector for introducing and expressing antisense nucleic acids is the adenovirus derived vector Adenop53TX. This vector expresses a herpes virus thymidine kinase (TX) gene for either positive or negative selection and an expression  
25 cassette for desired recombinant sequences such as antisense sequences. This vector can be used to infect cells including most cancers of epithelial origin, glial cells and other cell types. This vector as well as others that exhibit similar desired functions can be  
30 used to treat a mixed population of cells to selectively express the antisense sequence of interest. A mixed population of cells can include, for example, in vitro or ex vivo culture of cells, a tissue or a human subject.

35 Additional features may be added to the vector to ensure its safety and/or enhance its therapeutic efficacy. Such features include, for example, markers

that can be used to negatively select against cells infected with the recombinant virus. An example of such a negative selection marker is the TK gene described above that confers sensitivity to the antibiotic gancyclovir. Negative selection is therefore a means by which infection can be controlled because it provides inducible suicide through the addition of antibiotics. Such protection ensures that if, for example, mutations arise that produce mutant forms of the viral vector or antisense sequence, cellular transformation will not occur. Moreover, features that limit expression to particular cell types can also be included. Such features include, for example, promoter and expression elements that are specific for the desired cell type.

15       The present invention also provides methods for the selective destruction of defective mitochondria. Since the mitochondrial genome is heteroplasmic (i.e. it contains mutated and normal DNA), this will leave intact mitochondria carrying normal or wild-type DNA and these normal mitochondria will repopulate the targeted tissue, normalizing mitochondrial function. This can be accomplished by identifying unique characteristics of mitochondria carrying mutated DNA, designing a small molecule that is directed at one or more of these unique characteristics, and conjugating a mitochondrial toxin to this small molecule. Thus, a "targeting molecule" is any molecule that selectively accumulates in mitochondria having defective cytochrome c oxidase activity, and includes acridine orange derivatives and JC-1 derivatives as discussed hereinbelow.

30       "Mitochondrial toxins" are molecules that destroy or disable the selected mitochondria, and include phosphate, thiophosphate, dinitrophenol, maleimide and antisense oligonucleotides such as those discussed above. The toxin will be concentrated within the defective mitochondria by the targeting molecule and will disable or destroy selectively the defective



mitochondria. The molecule may be an active mitochondrial toxin in its conjugated form. However, it is preferred to design the molecule such that it is inactive in its conjugated form. The chemical linkage  
5 between the targeting molecule and the toxin may be a substrate for a mitochondria-specific enzyme or sensitive to redox cleavage. Choice of the linkage depends upon the chemical nature of the targeting molecule and toxin and the requirements of the cleavage  
10 process. Once the conjugate is concentrated in the defective mitochondria, the toxin is cleaved from the targeting molecule, activating the toxin.

Mitochondria with defective cytochrome c oxidase activity exhibit impaired electron transport, leading to  
15 decreased synthesis of adenosine triphosphate and general bioenergetic failure. As a consequence, mitochondria carrying mutated DNA will become enlarged and the intramitochondrial membrane potential increases.

Enlarged mitochondria have increased levels of  
20 cardiolipin and other negatively charged phospholipids. The acridine orange derivative 10N-nonylacridine orange (NAO) binds relatively specifically to cardiolipin and accumulates in dysfunctional mitochondria. The accumulation of NAO and other chemical derivatives of  
25 acridine orange, including but not limited to those with aliphatic chains of variable length attached to the ring nitrogen of acridine orange ([3,6-bis (dimethyl-amino) acridine]), such as 10N-pentylacridine orange, 10N-octylacridine orange, and dodecylacridine orange, is  
30 independent of the mitochondrial transmembrane potential. Maftah et al., Biochemical and Biophysical Research Communications 164 (1):185-190 (1989)). At concentrations up to 1  $\mu$ M, NAO and its derivatives can be used to target other molecules to the inner  
35 mitochondrial matrix. If the NAO is chemically linked to a mitochondrial toxin such as phosphate, thiophosphate, dinitrophenol, maleimide and antisense

oligonucleotides, then mitochondria accumulating the NAO-mitochondrial toxin conjugate can be selectively disabled or destroyed. Alternately, at high concentrations (3-10 $\mu$ M) NAO and its derivatives inhibit  
5 electron transport, ATP hydrolysis and P<sub>i</sub>-transport and disrupt respiration. (Maftah et al., FEBS Letters 260(2):236-240 (1990). At these concentrations, NAO is mitochondrial toxin.

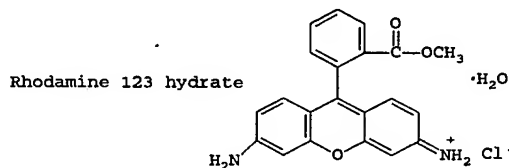
According to an embodiment of the present  
10 invention, the terminus of any aliphatic or other type of chain (such as polyethylene glycol) attached to the ring nitrogen of acridine orange is chemically derivatized with carboxylic acid, hydroxyl, sulfhydryl, amino or similar groups to accept any mitochondrial  
15 toxin. In other embodiments, additional sites of attachment of the mitochondrial toxin to acridine orange and acridine orange derivatives are selected. For example, the 10-N-(10-hydroxy-1-decyl)-3,6-bis(dimethylamino)acridine bromide salt may be prepared  
20 and further derivatized to 10-N-(10-phosphoryl-1-decyl)-3,6-bis(dimethylamino) acridine chloride salt or 10-N-(10-thiophosphoryl-1-decyl)-3,6-bis(dimethylamino)acridine chloride salt. Alternately, 10-N-(11-undecanoic acid)-3,6-bis(dimethylamino)acridine  
25 bromide salt may be prepared and further derivatized to 10-N-(11-undecan-1-oic acid 2,4-dinitrophenyl ester)-3,6-bis(dimethylamino) acridine bromide salt. Upon cleavage, the phosphate, thiphospate or dinitrophenol levels selectively increase within defective  
30 mitochondria and destroy them. The functionalization and covalent attachment of the toxin does not need to depend on subsequent release of the toxin by cleavage of the NAO from the toxin, if the attachment point on the toxin is non-interfering with the function of the toxin  
35 within the mitochondria.

Several examples of the preparation of acridine orange derivatives are summarized in Figure 4 and in

Examples IX(a)-IX(f) hereinbelow. Other modifications are permitted as known to those skilled in the art.

Still other embodiments of the present invention target changes in the intramitochondrial membrane potential due to defective cytochrome c oxidase activity. Delocalized lipophilic cations have been used to monitor mitochondrial membrane potential. The uptake of these cations is related to the presence of the negative sink inside the mitochondria created by the proton pump. As mitochondria increase in size due to cytochrome c oxidase defects, the transmembrane potential will increase and these defective mitochondria will accumulate lipophilic cations. According to an embodiment of the present invention, these lipophilic cations are conjugated to mitochondrial toxins and used to destroy defective mitochondria that possess increased transmembrane potentials. Rhodamine-123 the hydrated form of which is as follows:

20



has been used extensively to monitor mitochondrial membrane potential and can conjugate to mitochondrial toxins to concentrate toxins within the mitochondria. The compound 5,5',6, 6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazolo-carbocyanine iodide (JC-1) also accumulates in mitochondria dependent upon the transmembrane potential. When JC-1 exceeds a critical concentration, J-aggregates form in the mitochondrial matrix, and their size causes these JC-1 J-aggregates to diffuse slowly out of the mitochondria (Reers et al., Biochemistry, 30(18):4480-4486 (1991)). JC-1 may be chemically conjugated to a mitochondrial toxin, producing a long-lived toxic compound to mitochondria

displaying increased transmembrane potential relative to normal mitochondria.

As with NAO, by adding a functional group to the JC-1 structure one can covalently attach another chemical entity to the JC-1 subunit. Delivery to the cells then causes the dual agent to be preferentially transported into the mitochondria, where the dual agent may be cleaved at the covalent attachment to release a toxin within the mitochondria where it exerts the desired effect. Alternatively, the functionalization and covalent attachment of the toxin does not need to depend on subsequent release of the toxin by cleavage of the JC-1 from the active agent, if the attachment point on the active species is non-interfering with the function of the toxin within the mitochondria.

Figures 5, 6 and 7 outline the functionalization of JC-1 by several different methods. Examples IX(g)-IX(f) hereinbelow illustrate an oxygen functionality, but the same can be accomplished with a nitrogen, sulfur or carboxylic acid functionality.

By utilizing the quasi-symmetrical nature of JC-1, a new chemical entity may be synthesized that is "half" JC-1 and contains a functional group capable of being used as a point for covalent attachment of another chemical entity to the JC-1 subunit. The existence of the JC-1 subunit facilitates selective transport of the whole molecule to the mitochondria where, if desired, enzymes effect cleavage of the JC-1 subunit from the toxin, allowing it to exert the desired effect. Alternatively, the functionalization and covalent attachment of the toxin does not need to depend on subsequent release of the toxin by cleavage of the JC-1 subunit from the toxin, if the attachment point on the toxin is non-interfering with the function of the active agent within the mitochondria.

Figure 8 outlines the synthesis of a functionalized "half" JC-1 subunit by several different methods. The

attachment of the active chemical species is via the heteroatom incorporated in the JC-1 or "half" JC-1 structure. This attachment may be accomplished by any number of linking strategies such as by taking advantage  
5 of a functionality on the active molecule (such as a carboxylic acid to form an ester with the oxygen of the altered JC-1) or by using a linker to space between the JC-1 and the toxin. These strategies are well known to those skilled in the chemistry of preparing diagnostic  
10 or labelling molecules with reporter functions for biological studies and include ester, amide, urethane, urea, sulfonamide, and sulfonate ester (S.T. Smiley et al.; Proc. Nat'l. Acad. Sci. USA, 88:3671-3675 (1991)).

As noted hereinabove, mitochondria carrying mutated  
15 cytochrome c oxidase genes have increased levels of cardiolipin and other negatively charged phospholipids as well as increased mitochondrial membrane potential. As a result, the mitochondria selectively accumulate targeting molecules, including acridine orange  
20 derivatives and lipophilic cations such as rhodamine-123 and JC-1 derivatives. In addition to selectively introducing toxins into the mitochondria, such targeting molecules can also selectively introduce imaging ligands, which can form the basis of effective *in vivo*  
25 and *in vitro* diagnostic strategies. Such strategies include magnetic resonance imaging (MRI), single photon emission computed topography (SPECT), and positron emission tomography (PET). Preferred imaging ligands for the practice of the present invention include  
30 radioisotopes (such as  $^{123}\text{I}$ ,  $^{125}\text{I}$ ,  $^{18}\text{F}$ ,  $^{13}\text{N}$ ,  $^{15}\text{O}$ ,  $^{11}\text{C}$ ,  $^{99\text{m}}\text{Tc}$ ,  $^{67}\text{Ga}$  and so forth), haptens (such as digoxigenin), biotin, enzymes (such as alkaline phosphatase or horseradish peroxidase), fluorophores (such as fluorescein lanthanide chelates, or Texas Red®), and  
35 gadolinium chelates for MRI applications. Saha et al., Seminars in Nuclear Medicine, 4:324-349 (1994).

As an example of an *in vitro* diagnosis, a targeting molecule, such as an acridine orange or JC-1 derivative, is labelled with fluorescein as an imaging ligand. The labelled targeting molecule is introduced into a human  
5 tissue cell culture such as a primary fibroblast culture. After a period of several hours, cells having mitochondria with defective cytochrome c oxidase genes selectively absorb the labelled targeting molecule in amounts greater than cells without such mitochondria.  
10 The cells are then washed and sorted in a fluorescence activated cell sorter (FACS) such as that sold by Becton Dickinson. Threshold limits can be established for the FACS using cells with wild-type mitochondria. Similarly, in an *in vivo* diagnosis, a targeting molecule  
15 such as an acridine orange or JC-1 derivative is labelled with  $^{99m}\text{Tc}$ ,  $^{18}\text{F}$  or  $^{123}\text{I}$  as an imaging ligand. This labelled targeting molecule is introduced into the bloodstream of a patient. After a period of several hours, the labelled targeting molecule accumulates in  
20 those tissues having mitochondria with cytochrome-oxidase-defective genes. Such tissues can be directly imaged using positron-sensitive imaging equipment.

Selective destruction of defective mitochondria is also achieved by using ribozymes. Ribozymes are a class  
25 of RNA molecules that catalyze strand scission of RNA molecules independent of cellular proteins. Specifically, ribozymes may be directed to hybridize and cleave target mitochondrial mRNA molecules. The cleaved target RNA cannot be translated, thereby preventing  
30 synthesis of essential proteins which are critical for mitochondrial function. The therapeutic application thus involves designing a ribozyme which incorporates the catalytic center nucleotides necessary for function and targeting it to mRNA molecules which encode for  
35 dysfunctional COX subunits. The ribozymes may be chemically synthesized and delivered to cells or they can be expressed from an expression vector following

either permanent or transient transfection. Therapy is thus provided by the selective removal of mutant mRNAs in defective mitochondria.

5 Cellular and Animal Models for Diseases Associated with Mitochondrial Defects

Methods for depleting mitochondrial DNA ("mtDNA") from cells and then transforming those cells with mitochondria from other cells have been reported in the  
10 literature. King and Attardi, Science, 246:500-503 (1989), created human cells lacking mtDNA ( $\rho^0$ 206 -143B human osteosarcoma cells) and then repopulated these cells with mitochondria from foreign cells. Transformants with various mitochondrial donors  
15 exhibited respiratory phenotypes distinct from the host and recipient cells, indicating that the genotypes of the mitochondrial and nuclear genomes, or their interaction, play a role in the respiratory competence of cells. Chomyn et al. (Chomyn, A., et al., Mol. Cell  
20 Biol., 11:2236-2244 (1991)) repopulated  $\rho^0$ 206 cells with mitochondria derived from myoblasts of patients carrying MELAS-causing mutations in the mitochondrial gene for tRNA<sup>leu</sup>. The transformed cells were deficient in protein synthesis and respiration, mimicking muscle-biopsy cells  
25 from MELAS patients. More recently, Chomyn et al. (Chomyn, A., et al., Am. J. Hum. Genet., 54:966-974 (1994)) reported the use of blood platelets as a source of mitochondrial donors for repopulation of  $\rho^0$  cells.

However, the techniques for mitochondrial  
30 transformation of human cells described above allow only limited short term studies. Care has to be taken in growing cultures since transformed, undifferentiated cells containing wild-type mtDNA are healthier than those containing mutant mtDNA and therefore have a  
35 propagative advantage in culture. Over the course of several generations, cells with wild-type mtDNA would dominate the cellular population (i.e., mutant mtDNA

would be selected against) and cells containing mutated mtDNA would be lost.

In addition, the value of the previous cell lines is further limited because they are not of the same type as those cells in which pathogenesis of the disease is expressed. For example, Chomyn (Chomyn, A., et al., Am. J. Hum. Genet., 54:966-974 (1994)) used osteosarcoma cells as the recipient of mitochondria from cells of a MERRF patient. Yet the major impact of MERRF on patients is that it affects the brain and muscle to cause encephalomyopathy and myopathy. There is no known pathogenesis in bone cells.

The present invention overcomes these two serious limitations. First, by introducing mitochondria from diseased cells into an undifferentiated, immortal cell line, it is possible to maintain the transformants in culture almost indefinitely. Although it would be possible to study and use the undifferentiated cells themselves, it is preferred to take a sample of such cells, and then induce them to differentiate into the cell type that they are destined to become. For example, for neurodegenerative disease, cultures of primary neurons or neuroblastoma cell lines are preferred because these can be terminally differentiated after transfer of mtDNA with phorbol esters, growth factors and retinoic acid. Transfer of mtDNA into these cells results in cells that carry mutant mitochondrial mtDNA and which differentiate into post-mitotic cells with a neuronal or neuronal-like phenotype.

Post-mitotic cells with a neuronal phenotype have several advantages over other cells. Obviously, these cells are closer to the phenotype of cells affected in neurodegenerative disease. Since these cells are not actively dividing, the propagative advantage of cells containing wild-type mtDNA is not a significant problem during the test period (i.e., cells containing mutant mtDNA are not selected against in tissue cultures).



Also, when terminally differentiated, these cells are stable in culture. Post-mitotic cells accumulate mutant mtDNA over their life span in culture, resulting in enhanced bioenergetic failure with increasing time in culture. This leads to an exacerbation of mitochondrial dysfunction and alterations in biochemical events consistent with bioenergetic failure.

Thus, using  $\rho^0$  cells derived from cultures of primary neurons or neuroblastoma cell lines permits analysis of changes in the mitochondrial genome and closely mimics the functional effects of mitochondrial dysfunction in neurons and cells.

Mitochondria to be transferred to construct model systems in accordance with the present invention can be isolated from virtually any tissue or cell source. Cell cultures of all types could potentially be used, as could cells from any tissue. However, fibroblasts, brain tissue, myoblasts and platelets are preferred sources of donor mitochondria. Platelets are the most preferred, in part because of their ready abundance, and their lack of nuclear DNA. This preference is not meant to constitute a limitation on the range of cell types that may be used as donor sources.

Recipient cells useful to construct models in accordance with the present invention are undifferentiated cells of any type, but immortalized cell lines, particularly cancerous cell lines, are preferred, because of their growth characteristics. Many such cell lines are commercially available, and new ones can be isolated and rendered immortal by methods that are well known in the art. Although cultured cell lines are preferred, it is also possible that cells from another individual, e.g., an unaffected close blood relative, are useful; this could have certain advantages in ruling out non-mitochondrial effects. In any event, it is most preferred to use recipient cells that can be induced to differentiate by the addition of particular

chemical (e.g., hormones, growth factors, etc.) or physical (e.g., temperature, exposure to radiation such as U.V. radiation, etc.) induction signals.

It is most preferred that the recipient cells be selected such that they are of (or capable of being induced to become) the type that is most phenotypically affected in diseased individuals. For example, for constructing models for neurological diseases that are associated with mitochondrial defects, neuronal or neuroblastoma cell lines are most preferred.

In the examples below, mitochondria have been isolated by an adaptation of the method of Chomyn (Chomyn, A., et al., Am. J. Hum. Genet., 54:966-974 (1994)). However, it is not necessary that this particular method be used. Other methods, are easily substituted. The only requirement is that the mitochondria be substantially purified from the source cells and that the source cells be sufficiently disrupted that there is little likelihood that the source cells will grow and proliferate in the culture vessels to which the mitochondria are added for transformation.

In the examples, the mitochondrial DNA (mtDNA) of the target cells is removed by treatment with ethidium bromide. Presumably, this works by interfering with transcription or replication of the mitochondrial genome, and/or by interfering with mRNA translation. The mitochondria are thus rendered unable to replicate and/or produce proteins required for electron transport, and the mitochondria shut down, apparently permanently. However, it is important to note that it is not necessary for the purposes of this invention to use any particular method to remove the mitochondria or mitochondrial DNA.

Model systems made and used according to the present invention irrespective of whether the disease of interest is known to be caused by mitochondrial

disorders are equally useful where mitochondrial defects are a symptom of the disease, are associated with a predisposition to the disease, or have an unknown relationship to the disease. In addition, the use of  
5 model systems according to the present invention to determine whether a disease has an associated mitochondrial defect are within the scope of the present invention.

In addition, although the present invention is  
10 directed primarily towards model systems for diseases in which the mitochondria have metabolic defects, it is not so limited. Conceivably there are disorders wherein there are structural or morphological defects or anomalies, and the model systems of the present  
15 invention are of value, for example, to find drugs that can address that particular aspect of the disease. In addition, there are certain individuals that have or are suspected of having extraordinarily effective or efficient mitochondrial function, and the model systems  
20 of the present invention may be of value in studying such mitochondria. In addition, it may be desirable to put known normal mitochondria into cell lines having disease characteristics, in order to rule out the possibility that mitochondrial defects contribute to  
25 pathogenesis. All of these and similar uses are within the scope of the present invention, and the use of the phrase "mitochondrial defect" herein should not be construed to exclude such embodiments.

Determining the molecular switch that converts  
30 individuals from IGT to NIDDM would be of enormous medical significance. Having the ability to identify those individuals with a predisposition to convert from IGT to diabetes mellitus would be an advance in the diagnosis of late onset diabetes mellitus. Being able  
35 to prevent conversion of IGT to late onset diabetes mellitus would represent a major therapeutic advance.

Genetic defects in the mitochondrial genes encoding for components of the electron transport chain may be involved in the switch from IGT to NIDDM. These genetic defects may lead to perturbations of this protein complex and ultimately a drop in the production of adenosine triphosphate (ATP), the main source of fuel for cellular biochemical reactions.

When mitochondrial intracellular ATP levels drop, glucose transport into cells is impaired, metabolism of glucose is slowed and insulin secretion is decreased, all critical events in the switch from IGT to diabetes mellitus. Affected tissues are striated muscle (the major insulin-sensitive tissue) and pancreatic beta cells (insulin secreting cells). These target tissues contain non-dividing terminally differentiated cells that are susceptible to accumulation of mtDNA mutations. Achieving a threshold level of mutations in mtDNA in pancreatic beta cells could precipitate a drop in insulin secretion, providing a molecular mechanism for the switch in disease phenotype from IGT to diabetes mellitus. In addition, a similar mechanism may precipitate a loss of insulin responsivity in muscle.

Certain critical enzymes in the metabolism of glucose (hexokinases) and insulin secretion require ATP for proper function. Hexokinases and in particular glucokinase are bound to porin, a voltage dependent anion channel, located within the outer mitochondrial membrane. Porin, in turn, is apposed to the adenine nucleotide translocator of the inner mitochondrial membrane. Together these protein complexes form a conduit for delivery of ATP from the inner mitochondrial matrix to hexokinases bound to the outer membrane and for return of ADP generated by catalytic activity of these kinases. The ATP used by mitochondrial bound hexokinases is derived from the mitochondrial matrix and not the cytoplasm. Hexokinases require mitochondrial ATP for activation.

The foregoing and following description of the invention and the various embodiments is not intended to be limiting of the invention but rather is illustrative thereof. Those skilled in the art of molecular genetics can formulate further embodiments encompassed within the scope of the present invention.

#### EXAMPLES

##### Definitions of Abbreviations:

10

1 X SSC = 150 mM sodium chloride, 15 mM sodium citrate, pH 6.5-8

SDS = sodium dodecyl sulfate

BSA = bovine serum albumin, fraction IV

15

probe = a labelled nucleic acid, generally a single-stranded oligonucleotide, which is complementary to the DNA target immobilized on the membrane. The probe may be labelled with radioisotopes (such as <sup>32</sup>P), haptens (such as digoxigenin), biotin, enzymes (such as alkaline phosphatase or horseradish peroxidase), fluorophores (such as fluorescein or Texas Red), or chemilumiphores (such as acridine).

20

25

PCR = polymerase chain reaction, as described by Erlich et al., Nature 331:461-462 (1988) hereby incorporated by reference.

#### 30 Materials and methods

**Reagents.** Cell culture media were purchased from Gibco BRL (Gaithersburg, MD). 5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazolo-carbocyanine iodide (JC-1) and nonyl acridine orange were obtained from Molecular Bioprobes (Eugene, OR). Unless otherwise indicated, all other reagents were from Sigma Chemical Co. (St. Louis, Missouri).

35

**Cell Culture.** SH-SY5Y neuroblastoma cells (Biedler, J. L. et al., Cancer Res., 38:3751-3757 (1978)) were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum (FBS), penicillin (100 IU/ml), streptomycin (50  $\mu$ g/ml), glucose (4500 mg/ml), 25 mM HEPES, and glutamine (584 mg/ml) at 37°C in 5% CO<sub>2</sub>. In order to heat inactivate the FBS, it was thawed overnight at 4°C, warmed to 37°C, then heated to 56°C for 30 minutes.

10 DMEM was chosen over RPMI 1640 medium since RPMI is known to inhibit production of mitochondrial DNA (mtDNA) in depleted ( $\rho^0$ ) cell lines (Van Den Bogert, C. et al., J. of Cellular Physiol., 152:632-638 (1992)).

15 **Oxygen Consumption Measurements.** Cells were trypsinized from a 75 cm<sup>2</sup> flask, rinsed one time with HBSS (Hanks Balanced Salt Solution, Gibco BRL), resuspended at  $2.0 \times 10^7$  cell/ml in HBSS, and maintained at 37°C. An 80  $\mu$ l cell suspension sample was introduced into a Haas stirred polarographic microchamber (Haas, R. H. et al., Biochem. Med., 32:138-143 (1984)) in a final volume of 330  $\mu$ l in HBSS. Oxygen consumption was measured by a Yellow Springs Clark oxygen electrode No. 5531 and monitor No. 5300 (Yellow Springs, OH) at 37°C. Oxygen

20 utilization was calculated as described by Estabrook (Methods of Enzymol., 10:41-47 (1967)).

#### **Enzymatic Assays and Protein Determinations.**

Citrate synthase activity was determined using samples

30 of  $2 \times 10^5$  cells incubated at 30°C in a cuvette containing 0.04% triton X-100, 0.1 mM 5,5'-dithio-bis(2-nitrobenzoic) acid, 980  $\mu$ l of 100 mM tris pH 8.0 for 3 minutes prior to the assay. To initiate the reaction, 10  $\mu$ l of acetyl CoA and

35 oxaloacetic acid to final concentrations of 50  $\mu$ M and 500  $\mu$ M, respectively, were added. The cuvette was mixed by inversion and the increase in absorbance at 412 nm

was recorded for 2 to 3 minutes. The reaction is linear over this time period (Shepherd, D. et al, Methods in Enzymol., 13:11-16 (1969)). Complex IV (cytochrome c oxidase) and complex II (succinic dehydrogenase) activities were determined essentially as described (Parker, W. D., et. al., Neurology, 40:1302-1303 (1990)) except that cells ( $6 \times 10^5$  cells for COX activity and  $2 \times 10^5$  cells for succinic dehydrogenase) rather than isolated mitochondria were assayed, and membranes were lysed by incubation with n-dodecyl-beta-D-maltoside (0.2 mg/ml) for three minutes at 30°C prior to measurement of enzymatic rates. The assay reaction was initiated by the addition of reduced cytochrome c to the cuvette, which was inverted twice. The change in absorbance at 550 nm was measured continuously for 90 seconds. The fully oxidized absorbance value was determined by the addition of a few grains of ferricyanide to the cuvette. Rates were obtained at various cell concentrations to validate that the assay was in a linear range.

Non-enzymatic background activity was determined by pre-incubation of the cells with 1 mM potassium cyanide (KCN) prior to determination of the rate constant. Cyanide sensitive complex IV activity was calculated as a first-order rate constant after subtraction of background activity. Complex II activity was assayed by adding the cells to a cuvette containing assay buffer (10 mM succinate, 35 mM potassium phosphate, pH 7.2, 200  $\mu$ g/ml n-dodecyl-beta-D- maltoside, 1 mM KCN, 5 mM  $\text{MgCl}_2$ , 1  $\mu$ M rotenone and 1  $\mu$ M antimycin A). Assay volume was adjusted to a volume of 887 with assay buffer. After incubation at 30° C. for 10 minutes, 100  $\mu$ l of 0.6 mM 2,6-dichlorophenolindophenol (DCIP), as the final electron acceptor, was added for one minute for temperature equilibration. Three  $\mu$ l of a 20 mM solution of the synthetic ubiquinone analog, Q1 (Intermediate electron acceptor), was added to initiate the reduction of DCIP. The change in absorbance at 600nm for 1-3

minutes at 30°C was determined. Rates were obtained at various cell concentrations to validate that the assay was in a linear range. Background was determined by a repeat reaction in the presence of 10 mM malonate (competitive inhibitor). Specific complex II activity was calculated by subtracting malonate-inhibited background. All enzymatic activities were normalized to total cellular protein as determined by the Lowry method (J. Biol. Chem., 193:265-275 (1951)).

10

Complex I (NADH:ubiquinone oxidoreductase) activities were determined essentially as described in Parker, et al., Am. J. Neurol., 26:719-723 (1989), except that cells rather than isolated mitochondria were assayed.

15 Membranes were lysed by incubation of cells at  $2 \times 10^6$  cells/ml with 0.005 % digitonin in Hank's buffered salts plus 5 mM EDTA (HBSS/EDTA) for 20 seconds at 23°C. The solubilization was stopped by addition of 50 volumes of cold HBSS/EDTA. The lysed cells were centrifuged at  
20 14,000 g for 10 minutes at 4°C. The pellet was diluted to approximately 1 mg/ml protein in HBSS/EDTA with 1  $\mu$ M leupeptin, 1  $\mu$ M pepstatin and 100  $\mu$ M PMSF. Prior to complex I assays a 200  $\mu$ l aliquot of protein suspension in a 1.5 ml eppendorf tube was sonicated for 6 minutes  
25 in an ice packed cup horn sonicator (Heat Systems-Ultrasonics model W225) at 50 % duty cycle. The complex I assay reaction was initiated by the addition of 3  $\mu$ l of 20 mM ubiquinone-1 in ethanol to 10  $\mu$ l of 10mM NADH (in assay buffer), and 30-100  $\mu$ g of protein in a 1 ml  
30 total volume of assay buffer (25 mM potassium phosphate, pH 8.0, 0.25 mM EDTA, and 1.5 mM potassium cyanide) in a 1 ml cuvette that had been pre-incubated at 30° C for 3 minutes. The change in absorbance at 340 nm was measured for 120 seconds after which 5  $\mu$ l of 500  $\mu$ M  
35 rotenone in ethanol was added and the absorbance change was measured for another 120 seconds, to determine the rotenone sensitive Complex I activity. Complex I



activity was defined as the total rate (without rotenone) - total rate (with rotenone). The rates are calculated from the maximum linear portion of the curve using  $6.81 \text{ mM}^{-1}$  as the combined NADH-Q1 extinction

5 coefficient at 340 nm.

**Dye Uptake.** Cells were plated in 96 well microplates at  $4\text{-}50 \times 10^3$  cells/well overnight. Medium was decanted and the cells rinsed once with HBSS. The cells were  
10 incubated with  
5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazolo-carbocyanine iodide (JC-1,  $16 \mu\text{M}$ ) or nonyl acridine orange ( $1 \mu\text{g/ml}$ ) for sixty minutes at  $37^\circ\text{C}$ ., with  $\text{CO}_2$ , in a 100 nanoliter volume of HBSS. The medium was decanted  
15 and the cells rinsed three times with  $200 \mu\text{l}$  of HBSS and left in  $100 \mu\text{l}$  HBSS. Dye uptake was measured using a Millipore Cytofluor No. 2350 fluorescence measurement system (Bedford, MA). Filter sets used for JC-1 and nonyl acridine orange were 485 nm (excitation) and 530  
20 nm (emission). Bandwidths for the 485 nm, and 530 nm filters were 20 nm, and 25 nm respectively. Dye uptake by the cells was optimized for incubation time, concentration, and cell number, and shown to be linear with respect to cell number under the conditions chosen  
25 (manuscript in preparation). To define non-specific uptake of the mitochondrial membrane potential sensitive dye (JC-1), carbonyl cyanide m-chlorophenyl hydrazone (CCCP,  $5 \mu\text{M}$ ) was added concurrently with JC-1 to uncouple electron transport and dissipate the  
30 mitochondrial membrane potential (Johnson, L. V. et al., J. of Cell Biol., 88:526-535 (1981)).

In some experiments, dye uptake was also quantitated by fluorescence activated cell sorting (FACS-Scan, Becton-Dickinson) using dye concentrations  
35 and incubation times described above. Growing cells were trypsinized from a  $75 \text{ cm}^2$  flask, rinsed one time with PBS +  $1 \text{ mg/ml}$  glucose, resuspended in the same

buffer, split into separate tubes, treated and incubated with dye. After incubation, the cells were centrifuged at 200 X g for 10 minutes, the incubation medium was decanted, and the stained cells were resuspended in 2 ml of PBS + 1 mg/ml glucose and the cells were held on ice prior to FACS analysis. FACS analysis was carried out on  $1 \times 10^4$  cells with an excitation filter of 485 nm and an emission filter of 530 nm and a bandwidth of 42 nm.

- 10 **Slot blot analysis of mtDNA.** Total DNA from  $10^7$  SH-SY5Y parental and  $\rho^0$  cell isolates was isolated by a Qiagen Kit (Chatsworth, CA) and quantitated by absorbance at 260 nm and by agarose gel electrophoresis. Various amounts of total DNA were denatured by treatment with
- 15 0.2 N NaOH in 100  $\mu$ l volume at 65°C for 30 minutes. The sample was neutralized with 100  $\mu$ l of 2 M  $\text{NH}_4\text{OAc}$ . The DNA was vacuum blotted onto a Zeta probe membrane (Bio-Rad, Richmond, CA), and was wetted with 10X SSC (1.89 M sodium chloride, 188 mM sodium citrate, pH 7.0).
- 20 The membrane was then exposed to UV light (254 nm, 125 mJoule) and incubated with blocking buffer (0.2% I-Block, 0.5X SSC, 0.1% Tween-20) for 30 minutes at ambient temperature. The membrane was washed with hybridization buffer (5X SSC, 1% SDS, 0.5% BSA) in an
- 25 open small volume plastic dish.

Alkaline phosphatase-oligo conjugates were prepared as described by Ghosh (Bioconjugate Chem., 1:71-76 (1990)). Ten mls of hybridization buffer containing 2 pmol/ml of AP-oligo conjugate against the COX I subunit, specific for human mtDNA (CGTTTGGTATTGGGTATGGC), was

30 layered on the membrane and incubated for 60 minutes at 42°C. The membrane was washed three times with buffer 1 (1X SSC, 0.1% SDS, 5 minutes at RT), one time with buffer 2 (0.5X SSC, 0.1% SDS, three minutes at 50°C),

35 one time with buffer 3 (1X SSC, 1% triton X-100, three minutes at RT), one time with buffer 4 (1X SSC for ten minutes at RT) and finally one time briefly with

development buffer (50 mM NaHCO<sub>3</sub>, 1 mM MgCl<sub>2</sub>, pH 9.5). The membrane was developed with Lumi-phos (Boehringer Mannheim, Indianapolis, IN) as per manufactures procedures. To quantitate the mtDNA a standard curve of  
5 known quantities of plasmid containing the COX I gene was blotted at the same time.

#### EXAMPLE I

##### 10 Isolation and cloning of cytochrome c oxidase genes

DNA is obtained from AD patients and from non-Alzheimer's (normal) individuals. Age-matched normal individuals and AD patients classified as probable AD by NINCDS criteria (McKann et al., Neurology 34:939-944  
15 (1984)) are used.

For blood samples, 6 ml samples are drawn, added to 18 ml of dextrane solution (3% dextrane, average MW = 250,000 kiloDaltons (kDa), 0.9% sodium chloride, 1 mM ethylenedinitrilo tetraacetate, mixed and maintained at  
20 room temperature for 40 minutes without agitation to allow erythrocytes to sediment.

The plasma and leukocyte fraction is transferred to a centrifuge tube and leukocytes are collected by centrifugation at 14,000 x g for 5 minutes. The  
25 leukocyte pellet is resuspended in 3.8 ml of water and vortexed for 10 seconds to lyse remaining erythrocytes. 1.2 ml of 0.6 M sodium chloride is added and the sample is again centrifuged at 14,000 x g for 5 minutes to collect the leukocytes. The leukocyte pellet is  
30 resuspended in 0.4 ml of a solution containing 0.9% sodium chloride/1mM ethylenedinitrilo tetraacetate and stored at -80°C.

Total cellular DNA is isolated from 0.2 ml of the frozen leukocyte sample. The frozen leukocytes are  
35 thawed, then collected by centrifugation at 14,000 x g in a microcentrifuge for 5 minutes. The cell pellet is washed three times with 0.8 ml of Dulbecco's Phosphate

Buffered Saline (PBS; Gibco Laboratories, Life Technologies, Inc., Grand Island, N.Y.; catalog # 310-4040AJ) and resuspended in 0.3 ml water. The leukocytes are lysed by adding 0.06 ml of 10% sodium dodecyl sulfate to the cell suspension, then incubating the samples for 10 minutes in a boiling water bath. After the samples come to room temperature, cellular debris is pelleted by centrifugation at 14,000 x g for 5 minutes. The supernatant is transferred to a clean microcentrifuge tube and extracted twice with 0.5 ml of phenol:chloroform (1:1) and twice with chloroform. DNA is precipitated by addition of 0.03 ml of 5M sodium chloride and 0.7 ml of 100% ethanol to the sample. Following incubation at -80°C, the precipitated DNA is collected by centrifugation at 14,000 x g for 15 minutes. The DNA pellet is washed with 0.8 ml of 80% ethanol, briefly dried, then resuspended in 0.2-0.4 ml of TE buffer (10mM Tris-HCl, pH 7.5, 1 mM EDTA). The DNA concentration is determined by UV absorption at 260 nm.

As an alternative method for isolation of DNA from blood, 5 ml blood samples are drawn and added to Accuspin™ Tubes (12 ml or 50 ml capacity, Sigma Diagnostics, St. Louis, MO), prepared according to the manufacturer's instructions and containing Histopaque™ separation medium. The tubes are centrifuged at 1,000 x g for 10 minutes. The plasma and leukocyte fraction is transferred to a centrifuge tube containing 1 ml of TE buffer, and leukocytes are collected by centrifugation at 2,500 rpm for 10 minutes. The leukocyte pellet is resuspended in 5 ml TE buffer and 0.2 ml of 20% SDS and 0.1 ml of Proteinase K at 20 mg/ml are added. After incubation at 37°C for four hours while shaking the lysate is extracted twice with phenol and twice with chloroform:isoamyl alcohol (24:1). DNA is precipitated by addition of 1/10 volume 3.0 M sodium acetate (pH 5.0) and 2 volumes of ethanol. Following incubation at -20°C

overnight, the precipitated DNA is collected by centrifugation, washed with 70% ethanol, briefly dried, and resuspended in 0.1-0.2 ml of TE buffer. The DNA concentration is determined by UV absorption at 260 nm.

5 For brain samples, total cellular DNA is isolated from 0.1-0.2 grams of frozen brain tissue. The frozen brain tissue is placed into a glass dounce homogenizer (Pyrex, VWR catalog #7726-S) containing 3 ml of lysis buffer (50mM Tris-HCl, pH 7.9, 100 mM EDTA, 0.1 M NaCl,  
10 0.03 M dithiothreitol, 1% sodium dodecyl sulfate, 1 mg/ml proteinase K) and homogenized with a few strokes of the glass rod. The brain homogenate is transferred to an incubation tube and placed at 45-50°C for 30-60 minutes. After the addition of 5 ml of sterile water,  
15 the homogenate is extracted with phenol/chloroform two to three times, then twice with chloroform. DNA is precipitated by mixing the extracted sample with 1/20x volume of 5 M NaCl and 2.5x volumes of 200 proof ethanol and placed at -20°C. DNA is pelleted by centrifugation  
20 at 6,000 x g for 15 minutes. The DNA pellet is washed with 10ml of 80% ethanol, briefly dried, and resuspended in 200-400 µl of TE buffer. The DNA concentration is determined by UV absorption at 260 nm.

The target cytochrome c oxidase gene sequences are  
25 amplified by Polymerase Chain Reaction (PCR) (Erlich et al., Nature 331:461-462 (1988)). Primers are designed using the published Cambridge sequences for normal human COX genes. Primers are specific for COX gene sequences located approximately 100 nucleotides upstream and  
30 downstream of the mitochondrial COX genes encoding subunits I, II, and III. Primers have the following sequences: COX I-forward primer  
(5'-CAATATGAAATCACCTCGGAGC-3') (SEQ. ID. NO. 132), COX I- reverse primer (5'-TTAGCCTATAATTAACTTTGAC-3') (SEQ.  
35 ID. NO. 133), COX II-forward primer (5'-CAAGCCAACCCCATGGCCTCC-3') (SEQ. ID. NO. 134), COX II-reverse primer (5'-AGTATTTAGTTGGGGCATTTCAC-3') (SEQ.

ID. NO. 135), COX III-forward primer  
(5'-ACAATTCTAATTCTACTGACTATCC-3') (SEQ. ID. NO. 136),  
COX III-reverse primer (5'-TTAGTAGTAAGGCTAGGAGGGTG-3')  
(SEQ. ID. NO. 137).

5        Primers are chemically synthesized using a Cyclone  
Plus DNA Synthesizer (Millipore Corporation,  
Marlborough, MA) or a Gene assembler DNA Synthesizer  
(Pharmacia) utilizing beta-cyanoethylphosphoramidite  
chemistry. Newly synthesized primers are deprotected  
10 using ammonium hydroxide, lyophilized and purified by  
NAP-10 column chromatography (Pharmacia LKB  
Biotechnology Inc., Piscataway, NJ; catalog #  
17-0854-01). DNA concentration is determined by UV  
absorption at 260 nm.

15        Alternatively, primers are chemically synthesized  
using an ABL 394 DNA/RNA Synthesizer (Applied  
Biosystems, Inc., Foster City, CA) using standard beta-  
cyanoethylphosphoramidite chemistry. Without cleavage  
of the trityl group, the primers are deprotected with  
20 ammonium hydroxide and purified using Oligonucleotide  
Purification Cartridges (Applied Biosystems, Inc.,  
Foster City, CA). The DNA concentration is determined  
by UV absorption at 260 nm.

Amplification is performed using 0.5-1.0  $\mu$ g DNA in  
25 a reaction volume of 50-100  $\mu$ l containing 10mM Tris-HCl  
pH 8.3-9.5, 50 mM potassium chloride, 1-4 mM magnesium  
chloride, 200  $\mu$ M each of dATP, dCTP, dGTP, and dTTP  
("amplification cocktail"), 200 ng each of the  
appropriate COX forward and reverse primers and 5 units  
30 of AmpliTaq Polymerase (Perkin-Elmer Corporation;  
catalog # N801-0060).

Amplification using the GeneAmp PCR System 9600  
(Perkin Elmer Corporation) is allowed to proceed for one  
cycle at 95°C for 10 seconds, 25 cycles at 95°C for 1  
35 minute, 60°C for 1 minute, 72°C for 1 minute, one cycle  
at 72°C for 4 minutes, after which the samples are  
cooled to 4°C. Five separate amplification reactions

are performed for each patient and each cytochrome c oxidase subunit. After the reactions are complete, the samples for each patient and subunit are combined and the amplified product is precipitated at  $-80^{\circ}\text{C}$  by the  
5 addition 1/10 volume of 5 M sodium chloride and 2 volumes of 100% ethanol.

The PCR amplification product is pelleted by centrifugation, dried briefly, resuspended in 40  $\mu\text{l}$  of TE buffer and purified by agarose gel electrophoresis  
10 (Sambrook et al., "Molecular Cloning: A Laboratory Manual," Cold Spring Harbor Laboratory, 1988). DNA is stained with ethidium bromide and visualized under long wavelength UV light. Bands of the expected lengths (approximately 1,700 bp for COX I, 900 bp for COX II and  
15 1,000 bp for COX III) are excised from the gel. The gel containing the DNA is minced into small pieces and placed into a microcentrifuge tube. 0.3 ml of 1 M sodium chloride is added to the gel fragments and the sample is frozen at  $-80^{\circ}\text{C}$ , then thawed and incubated at  $50^{\circ}\text{C}$  for  
20 15-20 minutes. Agarose is sedimented by centrifugation at  $14,000 \times g$  for 5 minutes, the supernatant containing the DNA is transferred to a new vial and the DNA fragments are collected by ethanol precipitation.

The amplified DNA fragments are cloned into the  
25 plasmid pCRII (Invitrogen Corp., San Diego, CA) using the TA-Cloning Kit (Invitrogen Corp., San Diego, CA; catalog # K2000-01). Ligations are performed in a reaction volume of 11  $\mu\text{l}$  containing 1-5  $\mu\text{l}$  of PCR amplification product, 2  $\mu\text{l}$  of plasmid (50 ng), 1  $\mu\text{l}$  of  
30 10x ligation buffer and 1  $\mu\text{l}$  of T4 DNA Ligase (4 units). Ligation reactions are incubated at  $10-12^{\circ}\text{C}$  for 15-16 hours.

Vector-ligated PCR fragments are transformed into competent *E. coli* cells of the strains XL1-Blue MRF',  
35 XL2-Blue MRF' and SURE (Stratagene, San Diego, CA). Transformed cells are spread onto LB-agar plates containing ampicillin (50  $\mu\text{g/ml}$ ), kanamycin (50  $\mu\text{g/ml}$ ),

IPTG (isopropyl-3-D-thiogalactopyranoside, 20  $\mu$ g/ml) and X-Gal (100  $\mu$ g/ml). The blue/white color selection mechanism provided by the cloning vector in combination with the *E. coli* cells allows for easy detection of  
5 recombinant clones, which are white.

Multiple white colonies are selected for each patient and COX subunit and screened by PCR for the presence of a correct insert using nested primers derived from the published Cambridge sequences. The  
10 primers are specific for sequences located approximately 40-60 nucleotides upstream and downstream of COX genes encoding subunits I, II and III. The sequences of the primers are as follows: COX I-forward primer (5'-AGGCCTAACCCCTGTC-3') (SEQ. ID. NO. 138), COX  
15 I-reverse primer (5'-GGCCATGGGGTTGGC-3') (SEQ. ID. NO. 139), COX II-forward primer (5'-AGGTATTAGAAAAACCA-3') (SEQ. ID. NO. 140), COX II-reverse primer (5'-ATCTTTAACTTAAAAGG) (SEQ. ID. NO. 141), COX  
III-forward primer (5'-GCCTTAATCCAAGCC-3') (SEQ. ID. NO. 142), COX IIIreverse primer (5'-GAATGTTGTCAAACTAG-3')  
20 (SEQ. ID. NO. 143) .

DNA samples from lysed cell supernatants are used as templates for PCR amplification. Individual colonies are selected and incubated overnight at 37°C with  
25 shaking (225 rpm) in LB-broth containing ampicillin and kanamycin. 100-200  $\mu$ l of each culture is centrifuged at 14,000 x g for 2 minutes. The cell pellet is resuspended in 5-10  $\mu$ l of water, then lysed by incubation in a boiling water bath for 5 minutes.  
30 Cellular debris is removed by centrifugation at 14,000 x g for 2 minutes.

Amplification of the cloned DNA samples is performed in a reaction volume of 10  $\mu$ l containing amplification cocktail, 40 ng each of the appropriate  
35 COX-S forward and reverse primers and 0.25 units of AmpliTaq Polymerase. Amplification is performed for one cycle at 95°C for 10 seconds, 25 cycles at 95°C for 1



minute, 44°C for 1 minute, 72°C for 1 minute, and cooled to 4°C, using the GeneAmp PCR System 9600. PCR products are analyzed by horizontal agarose gel electrophoresis.

5

## EXAMPLE II

**Sequencing of cytochrome c oxidase (COX) genes**

Plasmid DNA containing the COX gene inserts is obtained as described in Example I is isolated using the Plasmid Quik™ Plasmid Purification Kit (Stratagene, San Diego, CA) or the Plasmid Kit (Qiagen, Chatsworth, CA, Catalog # 12145). Plasmid DNA is purified from 50 ml bacterial cultures. For the Stratagene protocol "Procedure for Midi Columns," steps 10-12 of the kit protocol are replaced with a precipitation step using 2 volumes of 100% ethanol at -20°C, centrifugation at 6,000 x g for 15 minutes, a wash step using 80% ethanol and resuspension of the DNA sample in 100 µl TE buffer. DNA concentration is determined by horizontal agarose gel electrophoresis, or by UV absorption at 260nm.

Sequencing reactions using double-stranded plasmid DNA are performed using the Sequenase Kit (United States Biochemical Corp., Cleveland, OH; catalog # 70770), the BaseStation T7 Kit (Millipore Corp.; catalog # MBBLSEQ01), the Vent Sequencing Kit (Millipore Corp; catalog # MBBLVEN01), the AmpliTaq Cycle Sequencing Kit (Perkin Elmer Corp.; catalog # N808-0110) and the Taq DNA Sequencing Kit (Boehringer Mannheim). The DNA sequences are detected by fluorescence using the BaseStation Automated DNA Sequencer (Millipore Corp.). For gene walking experiments, fluorescent oligonucleotide primers are synthesized on the Cyclone Plus DNA Synthesizer (Millipore Corp.) or the GeneAssembler DNA Synthesizer (Pharmacia LKB Biotechnology, Inc.) utilizing beta-cyanoethylphosphoramidite chemistry. The following primer sequences are prepared from the published Cambridge sequences of the COX genes for subunits I, II,

and III, with fluorescein (F; FluoreDite fluorescein amidite, Millipore Corp.; or FluorePrime fluorescein amidite, Pharmacia LKB Biotechnology, Inc.) being introduced in the last step of automated DNA synthesis:

5 COX I primer1 (5'-FAGGCCTAACCCCTGTC-3') (SEQ. ID. NO. 144); COX I primer2 (5'-FGTCACAGCCCATG-3') (SEQ. ID. NO. 145); COX I primer3 (5'-FCCTGGAGCCTCCGTAG-3') (SEQ. ID. NO. 146); COX I primer4 (5'-CTTCTTCGACCCCG-3') (SEQ. ID. NO. 147); COX I primer5 (5'-FCATATTTACCTCCG-3') (SEQ. ID. NO. 148); COX I primer6 (5'-FCCTATCAATAGGAGC-3') (SEQ. ID. NO. 149); COX I primer7 (5'-FCATCCTATCATCTGTAGG-3') (SEQ. ID. NO. 150); COX II primer1 (5'-FAGGTATTAGAAAAACCA-3') (SEQ. ID. NO. 151); COX II primer2 (5'-FTAACTAATACTAACATCT-3') (SEQ. ID. NO. 152); COX II primer3 (5'-FTGCGACTCCTTGAC-3') (SEQ. ID. NO. 153); COX III primer1 (5'-FGCCTTAATCCAAGCC-3') (SEQ. ID. NO. 154); COX III primer2 (5'-CAATGATGGCGCGATG-3') (SEQ. ID. NO. 155); COX III primer3 (5'-FCCGTATTACTCGCATCAGG-3') (SEQ. ID. NO. 156); COX III primer4 (5'-FCCGACGGCATCTACGGC-3') (SEQ. ID. NO. 157). Primers are deprotected and purified as described above. DNA concentration is determined by UV absorption at 260 nm.

Sequencing reactions are performed according to  
25 manufacturer's instructions except for the following modification: 1) the reactions are terminated and reduced in volume by heating the samples without capping to 94°C for 5 minutes, after which 4 µl of stop dye (3 mg/ml dextran blue, 95%-99% formamide; as formulated by  
30 Millipore Corp.) are added; 2) the temperature cycles performed for the AmpliTaq Cycle Sequencing Kit reactions, the Vent Sequencing kit reactions, and the Taq Sequence Kit consist of one cycle at 95°C for 10 seconds, 30 cycles at 95°C for 20 seconds, at 44°C for  
35 20 seconds and at 72°C for 20 seconds followed by a reduction in volume by heating without capping to 94°C for 5 minutes before adding 4 µl of stop dye.

Electrophoresis and gel analysis are performed using the BioImage and BaseStation Software provided by the manufacturer for the BaseStation Automated DNA Sequencer (Millipore Corp.). Sequencing gels are  
5 prepared according to the manufacturer's specifications. An average of ten different clones from each individual is sequenced. The resulting COX sequences are aligned and compared with published Cambridge sequences. Mutations in the derived sequence are noted and  
10 confirmed by resequencing the variant region.

As an alternative procedure for sequencing the COX genes, plasmid DNA containing the COX gene inserts obtained as described in Example I is isolated using the Plasmid Quik™ Plasmid Purification Kit with Midi Columns  
15 (Qiagen, Chatsworth, CA) Plasmid DNA is purified from 35 ml bacterial cultures. The isolated DNA is resuspended in 100  $\mu$ l TE buffer. DNA concentrations are determined by OD(260) absorption.

As an alternative method, sequencing reactions  
20 using double stranded plasmid DNA are performed using the Prism™ Ready Reaction DyeDeoxy™ Terminator Cycle Sequencing Kit (Applied Biosystems, Inc., Foster City, CA). The DNA sequences are detected by fluorescence using the ABI 373A Automated DNA Sequencer (Applied  
25 Biosystems, Inc., Foster City, CA). For gene walking experiments, oligonucleotide primers are synthesized on the ABI 394 DNA/RNA Synthesizer (Applied Biosystems, Inc., Foster City, CA) using standard beta-cyanoethylphosphoramidite chemistry. The following  
30 primer sequences are prepared from the published Cambridge sequences of the COX genes for subunits I, II, and III:

COX1 primer11 (5'-TGCTTCACTCAGCC-3') (SEQ. ID. NO. 158);  
COX1 primer1SF (5'-AGGCCTAACCCCTGTA-3') (SEQ. ID. NO. 159);  
35 COX1 primer11X (5'-AGTCCAATGCTTCACTCA-3') (SEQ. ID. NO. 160);  
COX1 primer12 (5'-GCTATAGTGGAGGC-3') (SEQ. ID. NO. 161);  
COX1 primer12A (5'-CTCCTACTCCTGCTCGCA-3') (SEQ. ID. NO. 162);  
COX1 primer12X (5'-TCCTGCTCGCATCTGCTA-3') (SEQ. ID. NO. 163);

- COX1 primer12XX (5'-CTCCTACTCCTGCTCGCA-3') (SEQ. ID. NO. 164);  
COX1 primer13 (5'-CCTACCAGGATTCG-3') (SEQ. ID. NO. 165);  
COX1 primer13A (5'-CCTACCAGGCTTCGGAA-3') (SEQ. ID. NO. 166);  
COX1 primer13X (5'-TCCTACCAGGCTTCGGAA-3') (SEQ. ID. NO. 167);  
5 COX1 primer14 (5'-CCTATCAATAGGAGC-3') (SEQ. ID. NO. 168);  
COX1 primer14XX (5'-GTCCTATCAATAGGAGCTGTA-3') (SEQ. ID. NO. 169);  
COX1 primer11C (5'-GTAGAGTGTGCAACC-3') (SEQ. ID. NO. 170);  
COX1 primer11CN (5'-GTCTACGGAGGCTCC-3') (SEQ. ID. NO. 171);  
COX1 primer11CX (5'-AGGTCTACGGAGGCTCCA-3') (SEQ. ID. NO. 172);  
10 COX1 primer11CXX (5'-AGGAGACACCTGCTAGGTGTA-3') (SEQ. ID. NO. 173);  
COX1 primer12C (5'-CCATACCTATGTATCC-3') (SEQ. ID. NO. 174);  
COX1 primer12CA (5'-TCACACGATAAACCTAGGAA-3') (SEQ. ID. NO. 175);  
COX1 primer12CX (5'-GACCATACCTATGTATCCAA-3') (SEQ. ID. NO. 176);  
COX1 primer13C (5'-CCTCCTATGATGGC-3') (SEQ. ID. NO. 177);  
15 COX1 primer13CN (5'-GTGTAGCCTGAGAATAGG-3') (SEQ. ID. NO. 178);  
COX1 primer13CXX (5'-GTCTAGGGTGTAGCCTGAGAA-3') (SEQ. ID. NO. 179);  
COX1 primer14C (5'-GGGTTGCGATTCTTCC-3') (SEQ. ID. NO. 180);  
COX1 primer14CN (5'-TGGATTGAAACCAGC-3') (SEQ. ID. NO. 181);  
COX1 primer14CX (5'-GTTGGCTTGAAACCAGCTT-3') (SEQ. ID. NO. 182);  
20 COX2 primer21 (5'-TCATAACTTTGTGTCGTC-3') (SEQ. ID. NO. 183);  
COX2 primer21N (5'-CATTTTCATAACTTTGTGTCGTC-3') (SEQ. ID. NO. 184);  
COX2 primer21NA (5'-AGGTATTAGAAAAACCA-3') (SEQ. ID. NO. 185);  
COX2 primer21NB (5'-AAGGTATTAGAAAAACC-3') (SEQ. ID. NO. 186);  
COX2 primer21X (5'-TTCATAACTTTGTGTCGTC-3') (SEQ. ID. NO. 187);  
25 COX2 primer2FSF (5'-AAGGTATTAGAAAAACC-3') (SEQ. ID. NO. 188);  
COX2 primer2SFA (5'-CCATGGCCTCCATGACTT-3') (SEQ. ID. NO. 189);  
COX2 primer22 (5'-TGGTACTGAACCTACG-3') (SEQ. ID. NO. 190);  
COX2 primer22A (5'-ACAGACGAGGTCAACGAT-3') (SEQ. ID. NO. 191);  
COX2 primer22X (5'-CATAACAGACGAGGTCAA-3') (SEQ. ID. NO. 192);  
30 COX2 primer21C (5'-AGTTGAAGATTAGTCC-3') (SEQ. ID. NO. 193);  
COX2 primer21CN (5'-TAGGAGTTGAAGATTAGTCC-3') (SEQ. ID. NO. 194);  
COX2 primer21CX (5'-TGAAGATAAGTCCGCCGTA-3') (SEQ. ID. NO. 195);  
COX2 primer22C (5'-GTTAATGCTAAGTTAGC-3') (SEQ. ID. NO. 196);  
COX2 primer22CXX (5'-AAGGTTAATGCTAAGTTAGCTT-3') (SEQ. ID. NO. 197);  
35 COX3 primer31 (5'-AAGCCTCTACCTGC-3') (SEQ. ID. NO. 198);  
COX3 primer31N (5'-CTTAATCCAAGCCTACG-3') (SEQ. ID. NO. 199);  
COX3 primer32 (5'-AACAGGCATCACCC-3') (SEQ. ID. NO. 200);  
COX3 primer32A (5'-CATCCGTATTACTCGCATCA-3') (SEQ. ID. NO. 201);  
COX3 primer31C (5'-GATGCGAGTAATACG-3') (SEQ. ID. NO. 202);  
40 COX3 primer31CX (5'-GATGCGAGTAATACGGAT-3') (SEQ. ID. NO. 203);  
COX3 primer32C (5'-AATTGGAAGTTAACGG-3') (SEQ. ID. NO. 204);  
COX3 primer32CX (5'-AATTGGAAGTTAACGGTA-3') (SEQ. ID. NO. 205);  
COX3 primer32CXX (5'-GTCAAACTAGTTAATTGGAA-3') (SEQ. ID. NO. 206);

Sequencing reactions are performed according to the manufacturer's instructions. Electrophoresis and sequence analysis are performed using the ABI 373A Data Collection and Analysis Software and the Sequence Navigator Software (ABI, Foster City, CA). Sequencing gels are prepared according to the manufacturer's specifications. An average of ten different clones from each individual is sequenced. The resulting COX sequences are aligned and compared with the published Cambridge sequence. Mutations in the derived sequence are noted and confirmed by sequence of the complementary DNA strand.

Mutations in each COX gene for each individual are compiled. Comparisons of mutations between normal and AD patients are made and summarized in Tables 1 and 2.

#### EXAMPLE III

##### **Detection of COX mutations by hybridization without prior amplification**

This example illustrates taking test sample blood, blotting the DNA, and detecting by oligonucleotide hybridization in a dot blot format. This example uses two probes to determine the presence of the abnormal mutation at codon 74 of the COX II gene (see Table 1) in mitochondrial DNA of Alzheimer's patients. This example utilizes a dot-blot format for hybridization, however, other known hybridization formats, such as Southern blots, slot blots, "reverse" dot blots, solution hybridization, solid support based sandwich hybridization, bead-based, silicon chip-based and microtiter well-based hybridization formats can also be used.

Sample Preparation Extracts and Blotting of DNA onto  
Membranes:

Whole blood is taken from the patient. The blood  
5 is mixed with an equal volume of 0.5-1 N NaOH, and is  
incubated at ambient temperature for ten to twenty  
minutes to lyse cells, degrade proteins, and denature  
any DNA. The mixture is then blotted directly onto  
10 prewashed nylon membranes, in multiple aliquots. The  
membranes are rinsed in 10 x SSC (1.5 M NaCl, 0.15 M  
Sodium Citrate, pH 7.0) for five minutes to neutralize  
the membrane, then rinsed for five minutes in 1 X SSC.  
For storage, if any, membranes are air-dried and sealed.  
In preparation for hybridization, membranes are rinsed  
15 in 1 x SSC, 1% SDS.

Alternatively, 1-10 mls of whole blood is  
fractionated by standard methods, and the white cell  
layer ("buffy coat") is separated. The white cells are  
lysed, digested, and the DNA extracted by conventional  
20 methods (organic extraction, non-organic extraction, or  
solid phase). The DNA is quantitated by UV absorption  
or fluorescent dye techniques. Standardized amounts of  
DNA (0.1-5  $\mu$ g) are denatured in base, and blotted onto  
membranes. The membranes are then rinsed.

25 Alternative methods of preparing cellular or  
mitochondrial DNA, such as isolation of mitochondria by  
mild cellular lysis and centrifugation, may also be  
used.

30 Hybridization and Detection:

For examples of synthesis, labelling, use, and  
detection of oligonucleotide probes, see  
"Oligonucleotides and Analogues: A Practical Approach",  
F. Eckstein, ed., Oxford University Press (1992); and  
35 "Synthetic Chemistry of Oligonucleotides and Analogs",  
S. Agrawal, ed., Humana Press (1993), which are  
incorporated herein by reference.

In this example two COX II codon 74 probes having the following sequences are used: ATC ATC CTA GTC CTC ATC GCC (SEQ. ID. NO. 14) (wild-type) and ATC ATC CTA ATC CTC ATC GCC (SEQ. ID. NO. 29) (mutant).

5 For detection and quantitation of the abnormal mutation, membranes containing duplicate samples of DNA are hybridized in parallel; one membrane is hybridized with the wild-type probe, the other with the AD probe. Alternatively, the same membrane can be hybridized  
10 sequentially with both probes and the results compared.

For example, the membranes with immobilized DNA are hydrated briefly (10-60 minutes) in 1 x SSC, 1% SDS, then prehybridized and blocked in 5 x SSC, 1% SDS, 0.5% casein, for 30-60 minutes at hybridization temperature  
15 (35-60°C, depending on which probe is used). Fresh hybridization solution containing probe (0.1-10 nM, ideally 2-3 nM) is added to the membrane, followed by hybridization at appropriate temperature for 15-60 minutes. The membrane is washed in 1 x SSC, 1% SDS, 1-3  
20 times at 45-60°C for 5-10 minutes each (depending on probe used), then 1-2 times in 1 x SSC at ambient temperature. The hybridized probe is then detected by appropriate means.

The average proportion of AD COX gene to wild-type  
25 gene in the same patient can be determined by the ratio of the signal of the AD probe to the normal probe. This is a semiquantitative measure of % heteroplasmy in the AD patient and can be correlated to the severity of the disease.

30 The above and other probes for alteration and quantitation of wild-type and mutant DNA samples are listed in Tables 4 and 5 hereinabove.

## EXAMPLE IV

**Detection of COX mutations by hybridization (without prior amplification)**A. Slot-blot detection of RNA/DNA with  $^{32}\text{P}$  probes

5

This example illustrates detection of COX mutations by slot-blot detection of DNA with  $^{32}\text{P}$  probes. The reagents are prepared as follows:

4xBP: 2% (w/v) Bovine serum albumin (BSA), 2% (w/v) polyvinylpyrrolidone (PVP, Mol. Wt.: 40,000) is dissolved in sterile  $\text{H}_2\text{O}$  and filtered through 0.22- $\mu$  cellulose acetate membranes (Corning) and stored at -20°C in 50-ml conical tubes.

DNA is denatured by adding TE to the sample for a final volume of 90  $\mu\text{l}$ . 10  $\mu\text{l}$  of 2 N NaOH is then added and the sample vortexed, incubated at 65°C for 30 minutes, and then put on ice. The sample is neutralized with 100  $\mu\text{l}$  of 2 M ammonium acetate.

A wet piece of nitrocellulose or nylon is cut to fit the slot-blot apparatus according to the manufacturer's directions, and the denatured samples are loaded. The nucleic acids are fixed to the filter by baking at 80°C under vacuum for 1 hr or exposing to UV light (254 nm). The filter is prehybridized for 10-30 minutes in ~5 mls of 1X BP, 5X SSPE, 1% SDS at the temperature to be used for the hybridization incubation. For 15-30-base probes, the range of hybridization temperatures is between 35-60°C. For shorter probes or probes with low G-C content, a lower temperature is used. At least  $2 \times 10^6$  cpm of detection oligonucleotide per ml of hybridization solution is added. The filter is double sealed in Scotchpak™ heat sealable pouches (Kapak Corporation) and incubated for 90 min. The filter is washed 3 times at room temperature with 5-minute washes of 20X SSPE : 3M NaCl, 0.02M EDTA, 0.2 Sodium Phosphate, pH 7.4, 1% SDS on a platform shaker. For higher stringency, the filter can be washed once at



the hybridization temperature in 1X SSPE, 1% SDS for 1 minute. Visualization is by autoradiography on Kodak XAR film at -70°C with an intensifying screen. To estimate the amount of target, compare the amount of target detected by visual comparison with hybridization standards of known concentration.

B. Detection of RNA/DNA by slot-blot analysis with alkaline phosphatase-oligonucleotide conjugate probes

This example illustrates detection of COX mutations by slot-blot detection of DNA with alkaline phosphatase-oligonucleotide conjugate probes, using either a color reagent or a chemiluminescent reagent. The reagents are prepared as follows:

Color reagent: For the color reagent, the following are mixed together, fresh 0.16 mg/ml 5-bromo-4-chloro-3-indolyl phosphate (BCIP), 0.17 mg/ml nitroblue tetrazolium (NBT) in 100 mM NaCl, 100 mM Tris. HCl, 5 mM MgCl<sub>2</sub> and 0.1 mM ZnCl<sub>2</sub>, pH 9.5.

Chemiluminescent reagent: For the chemiluminescent reagent, the following are mixed together, 250 µM 3-Adamantyl 4-methoxy 4-(2-phospho)phenyl dioxetane (AMPPD), (Tropix Inc., Bedford, MA) in 100 mM diethanolamine-HCl, 1 mM MgCl<sub>2</sub> pH 9.5, or prefomulated dioxetane substrate Lumiphos™ 530 (Lumigen, Inc., Southfield, MI).

DNA target (0.01-50 fmol) is immobilized on a nylon membrane as described above. The nylon membrane is incubated in blocking buffer (0.2% I-Block (Tropix, Inc.), 0.5X SSC, 0.1% Tween 20) for 30 min. at room temperature with shaking. The filter is then prehybridized in hybridization solution (5X SSC, 0.5% BSA, 1% SDS) for 30 minutes at the hybridization temperature (37-60°C) in a sealable bag using 50-100 µl of hybridization solution per cm of membrane. The

solution is removed and briefly washed in warm hybridization buffer. The conjugate probe is then added to give a final concentration of 2-5 nM in fresh hybridization solution and final volume of 50-100  $\mu\text{l}/\text{cm}^2$  of membrane. After incubating for 30 minutes at the hybridization temperature with agitation, the membrane is transferred to a wash tray containing 1.5 ml of preheated wash-1 solution (1X SSC, 0.1% SDS)/ $\text{cm}^2$  of membrane and agitated at the wash temperature (usually optimum hybridization temperature minus 10°C) for 10 minutes. Wash-1 solution is removed and this step is repeated once more. Then wash-2 solution (1X SSC) added and then agitated at the wash temperature for 10 minutes. Wash-2 solution is removed and immediate detection is done by color.

Detection by color is done by immersing the membrane fully in color reagent, and incubating at 20-37°C until color development is adequate. When color development is adequate, the development is quenched by washing in water.

For chemiluminescent detection, the following wash steps are performed after the hybridization step (see above). Thus, the membrane is washed for 10 min. with wash-1 solution at room temperature, followed by two 3-5 min. washes at 50-60°C with wash-3 solution (0.5X SSC, 0.1% SDS). The membrane is then washed once with wash-4 solution (1X SSC, 1% Triton X 100) at room temperature for 10 min., followed by a 10 min. wash at room temperature with wash-2 solution. The membrane is then rinsed briefly (~1 min.) with wash-5 solution (50mM  $\text{NaHCO}_3$ /1mM  $\text{MgCl}_2$ , pH 9.5).

Detection by chemiluminescence is done by immersing the membrane in luminescent reagent, using 25-50  $\mu\text{l}$  solution/ $\text{cm}^2$  of membrane. Kodak XAR-5 film (or equivalent; emission maximum is at 477 nm) is exposed in a light-tight cassette for 1-24 hours, and the film developed.

## EXAMPLE V

**Detection of COX mutations by amplification and hybridization**

This example illustrates taking a test sample of blood, preparing DNA, amplifying a section of a specific COX gene by polymerase chain reaction (PCR), and detecting the mutation by oligonucleotide hybridization in a dot blot format.

10 Sample Preparation and Preparing of DNA:

Whole blood is taken from the patient. The blood is lysed, and the DNA prepared for PCR by using procedures described in Example I.

15 Amplification of Target COX genes by Polymerase Chain Reaction, and Blotting onto Membranes:

The treated DNA from the test sample is amplified using procedures described in Example I. After amplification, the DNA is denatured, and blotted directly onto prewashed nylon membranes, in multiple aliquots. The membranes are rinsed in 10 x SSC for five minutes to neutralize the membrane, then rinsed for five minutes in 1 X SSC. For storage, if any, membranes are air-dried and sealed. In preparation for hybridization, membranes are rinsed in 1 x SSC, 1% SDS.

Hybridization and Detection:

Hybridization and detection of the amplified genes are accomplished as detailed in Example III.

Although the invention has been described with reference to the disclosed embodiments, those skilled in the art will readily appreciate that the specific examples provided herein are only illustrative of the invention and not limitative thereof. It should be understood that various modifications can be made without departing from the scope of the invention.

## EXAMPLE VI

**Synthesis of Antisense Oligonucleotides**

Standard manufacturer protocols for solid phase phosphoramidite-based DNA or RNA synthesis using an ABI  
5 DNA synthesizer are employed to prepare antisense oligomers. Phosphoroamidite reagent monomers (T, C, A, G, and U) are used as received from the supplier. Applied Biosystems Division/Perkin Elmer, Foster City, CA. For routine oligomer synthesis, 1 $\mu$ mole scale  
10 syntheses reactions are carried out utilizing THF/I<sub>2</sub>/lutidine for oxidation of the phosphoramidite and Beaucage reagent for preparation of the phosphorothioate oligomers. Cleavage from the solid support and deprotection are carried out using ammonium hydroxide  
15 under standard conditions. Purification is carried out via reverse phase HPLC and quantification and identification is performed by UV absorption measurements at 260nm, and mass spectrometry.

20

## EXAMPLE VII

**Inhibition of Mutant Mitochondria in Cell Culture**

Antisense phosphorothioate oligomer complementary to the COX gene mutant at codon 193 and thus non-complementary to wild-type COX gene mutant RNA is added  
25 to fresh medium containing Lipofectin® Gibco BRL (Gaithersburg, MD) at a concentration of 10  $\mu$ g/ml to make final concentrations of 0.1, 0.33, 1, 3.3, and 10  $\mu$ M. These are incubated for 15 minutes then applied to the cell culture. The culture is allowed to incubate  
30 for 24 hours and the cells are harvested and the DNA isolated and sequenced as in previous examples. Quantitative analysis results shows a decrease in mutant COX DNA to a level of less than 1% of total COX.

The antisense phosphorothioate oligomer non-  
35 complementary to the COX gene mutant at codon 193 and non-complementary to wild-type COX is added to fresh medium containing lipofectin at a concentration of 10

$\mu\text{g/mL}$  to make final concentrations of 0.1, 0.33, 1, 3.3, and 10  $\mu\text{M}$  these are incubated for 15 minutes then applied to the cell culture. The culture is allowed to incubate for 24 hours and the cells are harvested and  
5 the DNA isolated and sequenced as in previous examples. Quantitative analysis results showed no decrease in mutant COX DNA.

#### EXAMPLE VIII

##### 10 Inhibition of Mutant Mitochondria in Vivo

Mice are divided into six groups of 10 animals per group. The animals are housed and fed as per standard protocols. To groups 1 to 4 is administered ICV, antisense phosphorothioate oligonucleotide, prepared as  
15 described in Example VI, complementary to mutant COX gene RNA, respectively 0.1, 0.33, 1.0 and 3.3 nmol each in 5  $\mu\text{L}$ . To group 5 is administered ICV 1.0 nmol in 5  $\mu\text{L}$  of phosphorothioate oligonucleotide non-complementary to mutant COX gene RNA and non-complementary to wild-type  
20 COX gene RNA. To group 6 is administered ICV vehicle only. Dosing is performed once a day for ten days. The animals are sacrificed and samples of brain tissue collected. This tissue is treated as previously described and the DNA isolated and quantitatively  
25 analyzed as in previous examples. Results show a decrease in mutant COX DNA to a level of less than 1% of total COX for the antisense treated group and no decrease for the control group.

30

#### EXAMPLE IX

##### Agents for the Detection and

##### Selective Destruction of Defective Mitochondria

##### a. Preparation of 10-N-(10-Hydroxy-1-decyl)-3,6-bis(dimethylamino)acridine bromide salt

35 3,6-bis(dimethylamino)acridine (1.0 millimole) is dissolved in DMF (100 mL) containing 1.1 equivalent of tertiary amine base. To this is added 10-hydroxy-1-

bromo decane (1.1 millimole), and the mixture is heated to reflux. When monitoring by TLC shows no remaining 3,6-bis(dimethylamino)acridine, the reaction is cooled and the 10-N-(10-hydroxy-1-decyl)-3,6-bis(dimethylamino)acridine is isolated (0.75 millimoles).

b. Preparation of 10-N-(10-phosphoryl-1-decyl)-3,6-bis(dimethylamino)acridine chloride salt  
10 10-N-(10-Hydroxy-1-decyl)-3,6-bis(dimethylamino)acridine (1.0 millimole) is dissolved in pyridine (100 mL). To this is added 2-(N,N-dimethylamino)-4-nitrophenyl phosphate (1.1 millimole) according to the procedure of Taguchi (Chem. Pharm. Bull., 23:1586 (1975), and the mixture is stirred under  
15 a nitrogen atmosphere. When monitoring by TLC showed no remaining 10-N-(10-hydroxy-1-decyl)-3,6-bis(dimethylamino)acridine, the reaction is worked up according to Taguchi and the 10-N-(10-phosphoryl-1-decyl)-3,6-bis(dimethylamino)acridine is isolated (0.75  
20 millimoles).

c. Preparation of 10-N-(10-thiophosphoryl-1-decyl)-3,6-bis(dimethylamino)acridine chloride salt  
25 10-N-(10-hydroxy-1-decyl)-3,6-bis(dimethylamino)acridine (1.0 millimole) is dissolved in DMF (100 mL). To this is added triimidazolyl-1-phosphine sulfide (1.1 millimole) according to the procedure of Eckstein (Journal of the American Chemical Society, 92:4718, (1970)) and the mixture stirred under  
30 a nitrogen atmosphere. When monitoring by TLC shows no remaining 10-N-(10-Hydroxy-1-decyl)-3,6-bis(dimethylamino)acridine, the reaction is worked up according to Eckstein and the 10-N-(10-thiophosphoryl-1-decyl)-3,6-bis(dimethylamino)acridine is isolated (0.75  
35 millimoles).

d. Preparation of 10-N-(11-undecanoic acid)-3,6-bis(dimethylamino)acridine bromide salt

3,6-Bis(dimethylamino)acridine (1.0 millimole) is dissolved in DMF (100 mL). To this is added 11-bromo undecanoic acid (1.1 millimole) and the mixture is heated to reflux. When monitoring by TLC shows no remaining 3,6-bis(dimethylamino)acridine, the reaction is cooled and the 10-N-(11-undecanoic acid)-3,6-bis(dimethylamino)acridine is isolated (0.75 millimoles).

e. Preparation of 10-N-(11-undecyl-2,4-dinitrophenyl urethane)-3,6-bis(dimethylamino)acridine bromide salt

10-N-(11-Undecanoic acid)-3,6-bis(dimethylamino)acridine (1.0 millimole) is dissolved in THF (100 mL). To this is added 2,4-dinitrophenol (1.1 millimole) and diphenylphosphoryl azide (1.1 millimole), and the mixture is stirred while heating to 70°C. When monitoring by TLC shows no remaining 10-N-(11-undecanoic acid)-3,6-bis(dimethylamino)-acridine, the reaction is cooled and the 10-N-(11-undecyl-2,4-dinitrophenyl urethane)-3,6-bis(dimethylamino)acridine is isolated (0.75 millimoles).

f. Preparation of 10-N-(11-undecan-1-oic acid 2,4-dinitrophenyl ester)-3,6-bis(dimethylamino)acridine bromide salt

10-N-(11-undecanoic acid)-3,6-bis(dimethylamino)acridine (1.0 millimole) is dissolved in DMF (100 mL). To this is added 2,4-dinitrophenol (1.1 millimole), dicyclohexylcarbodiimide (1.1 millimole) and hydroxybenztriazole (1.1 millimole), and the mixture is stirred. When monitoring by TLC shows no remaining 10-N-(11-undecanoic acid)-3,6-bis(dimethylamino)-acridine, the reaction is cooled and the 10-N-(11-undecan-1-oic acid 2,4-dinitrophenyl ester)-3,6-

bis(dimethylamino)acridine is isolated (0.75 millimoles).

g. Preparation of N'-(2-hydroxyethyl)-JC-1

5 According to the procedure of Yamamoto et al. Bulletin of the Chemical Society of Japan, 46:1509-11 (1973), 2-methyl-5,6-dichloro-N-ethyl-N'-(2-hydroxyethyl) benzimidazole is heated with aniline and ethyl orthoformate at 100°C. To this is added acetic  
10 anhydride and potassium acetate and heating is continued at 160°C. The reaction is worked up as described in Yamamoto et al. and the product isolated.

h. Preparation of bis N'-(2-phosphoryl-1-ethyl)-JC-1

15 N'-(2-hydroxyethyl)-JC-1 (1.0 millimole) is dissolved in pyridine (100 mL). To this is added 2-(N,N-dimethylamino)-4-nitrophenyl phosphate (1.1 millimole) according to the procedure of Taguchi (Chem. Pharm. Bull., 23, 1586 (1975), and the mixture is  
20 stirred under a nitrogen atmosphere. When monitoring by TLC shows no remaining 10-N-(10-hydroxy-1-decyl)-3,6-bis(dimethylamino)acridine, the reaction is worked up according to Taguchi and bis N'-(2-phosphoryl-1-ethyl) JC-1 was isolated (0.75 millimoles).

25

EXAMPLE X.

**Preparation of immortalized  $\rho^0$  cell lines**

In order to produce cell lines expressing mitochondrial-DNA mutations that could be propagated and  
30 maintained in an undifferentiated state, and which could then undergo terminal differentiation, neuroblastoma cells were depleted of mitochondrial DNA, and mitochondria isolated from platelets of an AD patient were placed into those cells.

35 In order to convert them into  $\rho^0$  cells, SH-SY5Y neuroblastoma cells (Biedler, J. L. et al., Cancer Res., 38:3751-3757 (1978)) were cultured in the presence of



ethidium bromide for varying periods of time (30-70 days) and at varying concentrations (0.01 to 5  $\mu\text{g/ml}$ ). The cells were passaged every week, and the media was changed every 3 days. Ethidium bromide concentrations higher than these resulted in cell death after 2 to 3 weeks. A noticeable fall off in growth rate occurred at approximately 33 days. Cell lines chosen for further study were exposed to the various concentrations for either 33 or 64 days. Cell lines treated for 33 days, 45 days or 64 days 5.0  $\mu\text{g/ml}$  ethidium bromide (EtBr) were designated  $\rho^\circ$  33/5,  $\rho^\circ$  45/5 and  $\rho^\circ$  64/5, respectively.

Production of respiration deficient mutants was monitored by cyanide inhibitable  $\text{O}_2$  utilization. It was observed that oxygen utilization declined as a function of time and ethidium bromide concentration and was undetectable after 64 days of exposure to 5.0  $\mu\text{g/ml}$  concentration of ethidium bromide, as shown in FIG. 9 (see also Table 6). Oxygen utilization was determined polarographically in cells treated for either 33 days (closed circles) or 64 days (open circles) with varying concentrations of EtBr. Nonspecific  $\text{O}_2$  consumption was determined in the presence of 1 mM KCN and was subtracted from measured total rates. Data are shown S.E.M. for at least 2 independent experiments.

The effectiveness of ethidium bromide in shutting down electron transport was confirmed by treating cells for 64 days with EtBr at various concentrations, and then measuring oxygen consumption in the presence of specific inhibitors of complex I (rotenone), complex III (antimycin), and complex IV (cyanide). As shown in FIG. 10, treatment with ethidium bromide at 5  $\mu\text{g/ml}$  resulted in suppression of virtually all oxygen utilization that was sensitive to either complex I inhibition or complex III inhibition (FIG. 10). Data are shown S.E.M. for at least 2 independent experiments. Complex II enzyme activity was less perturbed, since none of its subunits

are encoded by mitochondrial DNA, and its proteins are apparently normally transported and inserted into the enlarged mitochondria of  $\rho^0$  cells in a functional state. Similarly, activity of the mitochondrial matrix enzyme citrate synthase, was comparable in parental and  $\rho^0$  SH-SY5Y cells, but decreased by approximately 50% in  $\rho^0$  64/5 cells. The unresponsiveness of this enzyme is not surprising since it is encoded by nuclear genes, and its expression should not be affected by mtDNA depletion.

It apparently is also normally transported and inserted into the enlarged mitochondria of  $\rho^0$  cells in a functional state. These findings are confirmed by direct measurement of the activities of complex IV, Complex II, and citrate synthetase (Table 6); note that complex IV activity for both the  $\rho^0$  64/5 cells and  $\rho^0$  33/5 cells were virtually undetectable.

$\rho^0$  cells were cultured in SH-SY5Y medium supplemented with uridine (50  $\mu\text{g/ml}$ ) and pyruvate (100  $\mu\text{g/ml}$ ) in order to support growth (King, M. P. et al., Science, 246:500-503 (1989)). As shown in FIG. 11,  $\rho^0$  64 days/5  $\mu\text{g/ml}$  ethidium bromide (" $\rho^0$  64/5") propagated in the presence of 100  $\mu\text{g/ml}$  pyruvate (closed squares) or both pyruvate and uridine (open circles) thrived, but  $\rho^0$  64/5 cells placed in media containing 50  $\mu\text{g/ml}$  uridine (open squares) or no addition (open triangles) did not. Parental SH-SY5Y (solid circles) were grown as a positive control with no addition (solid circles), and not surprisingly, grew the best. Each point represents the average cell number/well of triplicate wells in a 24 well plate. The generation time for SH-SY5Y was 24 hours in normal medium; this compared to 48 hours for  $\rho^0$  64/5 cells in medium containing pyruvate. The  $\rho^0$  64/5 cells reached the same final density as the parental line. Uridine alone, like cells that received no addition, did not support growth of  $\rho^0$  cells and cell death was noted after three days of plating.

The ability of pyruvate alone to support growth indicates that the enzyme dihydroorotate dehydrogenase, essential to de novo synthesis of uridine, may still be active in  $\rho^0$  cells of the present invention. Depletion  
5 of mtDNA has been shown to cause uridine auxotrophy in other  $\rho^0$  cell isolates (King et al., Science, 246:500-503 (1989)).

Rates of reversion from the  $\rho^0$  phenotype were determined by plating  $2 \times 10^6$  cells in a 75 cm<sup>2</sup> flask and  
10 culturing in uridine/pyruvate deficient selection medium. The viability dependence on uridine and pyruvate appeared within 2-3 weeks when most cells died. The very few surviving cells were then sub-cultured and designated as revertants. Reversion frequency as  
15 measured by survival under these conditions was  $1 \times 10^{-5}$  for  $\rho^0$  33/5 clones and  $1 \times 10^{-6}$  for  $\rho^0$  64/5 at 3 weeks (Table 6). The very few surviving cells were subcultured. Activities of complex II, complex IV, citrate synthase and O<sub>2</sub> utilization returned to control  
20 levels in these subcultures, indicating that estimation of reversion by survival in selection medium was paralleled by return of ETC activity (Table 6).

Table 6

Respiratory and Biochemical Activities in Parental and  $\rho^0$  Cells

Cell	O <sub>2</sub> consumption nmol/min-mg (S.D.)	Complex IV min-mg <sup>-1</sup> (S.D.)	Complex II min-mg <sup>-1</sup>	Citrate Synthase min-mg <sup>-1</sup>	Reversion Rate
SH-SY5Y	3.25 (0.57)	2.025 (0.052)	28.49	174.4	
$\rho^0$ 33/5	0.21 (0.26)	0.008 (0.003)	30.69	158.2	10 <sup>-5</sup>
$\rho^0$ 33/5 revertant	3.72 (0.77)	5.490 (0.281)	29.58	167.4	
$\rho^0$ 64/5	0.00 (0.28)	0.048 (0.038)	7.20	83.2	10 <sup>-6</sup>

Note. All activities are normalized to total cellular protein in mg.

Binding of the fluorescent dye nonylacridine orange, was greatly increased in SH-SY5Y cells as a function of ethidium bromide exposure for 64 days, as shown in FIG. 12. Assay was performed in 96 well microplates; cells were plated at  $2 \times 10^4$  cells per well 24 hours prior to the addition of  $1 \mu\text{g/ml}$  nonyl acridine orange. Measurements were made as described above. Data are shown as the mean of 8 experiments  $\pm$  the standard deviation. Since nonylacridine orange binds selectively to cardiolipin, an inner mitochondrial membrane lipid, its uptake correlates with the number and size of the mitochondria (Leprat, P., et al., Exp. Cell Res., 186:130-137 (1990)). The data shown in FIG. 12 suggest that the ethidium bromide treated cells have increasing quantities of inner mitochondrial membrane, which would be expected, since cells lacking mitochondrial DNA have been observed to have large, irregular mitochondria (Morais, R., et al., In Vitro Cell. and Devel. Biol., 21:649-658 (1988)).

Similarly, as shown in FIG. 13, binding of the cationic dye JC-1 was also increased in ethidium bromide-treated cells. Measurements were made by fluorescent plate reader in 96 well microplates as described using  $16 \mu\text{M}$  JC-1, and non-specific uptake was measured by concurrent addition of  $5 \mu\text{M}$  CCCP. Cells were plated at  $2 \times 10^4$  cells per well 24 hours prior to the addition of dye, and measurements were made as described above. Data are shown as the mean of 8 experiments  $\pm$  SD. Since JC-1 is known to equilibrate across the mitochondrial membrane as a function of the transmembrane electrical potential (Ehrenberg, B., et al., Biophysical J., 53:785-794 (1988)), the data shown in FIG. 13 indicate that the enlarged mitochondria expected in cells lacking mtDNA are able to establish increased transmembrane proton gradients, despite the lack of mitochondrial DNA, and despite the resulting lack of complex IV activity. This is consistent with

the observed increase of nonyl acridine orange uptake (see above).

As a final further confirmation that the  $\rho^0$  cells lack mtDNA, total DNA was extracted from untreated SH-SY5Y cells or SH-SY5Y cells exposed to ethidium bromide for 64 days at a concentration of 5  $\mu\text{g/ml}$ , and mtDNA was analyzed by Southern blotting. Equal amounts of DNA were separated on agarose gels, transferred to nitrocellulose membranes, and hybridized with  $^{32}\text{P}$ -labelled mtDNA specific probe. SH-SY5Y  $\rho^0$  cells had less than one mtDNA/cell when compared to a standard curve based on the known quantities of COX I gene (data not shown). This is essentially a finding of no detectable mtDNA, establishing conclusively that these cells were in the  $\rho^0$  state.

The foregoing results suggest that the concentration of EtBr used to achieve the  $\rho^0$  phenotype appears to be cell type specific. The parental neuroblastoma cell line needed high doses of EtBr (5  $\mu\text{g/ml}$ ) for long periods to induce the  $\rho^0$  phenotype; dosages needed were 10 and 100 times greater than that need to produce  $\rho^0$  fibroblast (Leprat, P., et al., Exp. Cell Res., 186:130-137 (1990)) and  $\rho^0$  osteosarcoma cells (King et al., Science, 246:500-503 (1989)), respectively. SH-SY5Y cells may have high resistance to EtBr-induced toxicity. Of course, titrating the amount of ethidium bromide and the time needed for a given new type of cell is well within the average skill in the art.

It is important to note that in the case of neuroblastoma cells, once the  $\rho^0$  phenotype appeared, continued treatment was necessary to obtain an acceptably low reversion rate, reversion being defined as the reappearance of the wild type phenotype when  $\rho^0$  cells are grown without supplemented pyruvate. High reversion rates of  $\rho^0$  cells fused with donor platelets would result in false positives during cybrid colony

selection. An acceptable level of less than one reversion in  $10^6$  was achieved after 64 days of EtBr treatment. Again, determining what duration of treatment is needed should be well within the average skill in the art.

#### EXAMPLE XI

##### Differentiation of the immortal $\rho^0$ cells

The  $\rho^0$  cells were induced to differentiate using phorbol ester (12-O-tetradecanoylphorbol-13-acetate, TPA) or growth factors. After two weeks of treatment with  $16 \mu\text{M}$  TPA or  $1 \mu\text{M}$  retinoic acid, the  $\rho^0$  cells expressed long neurites with secretory granules typical of differentiating neuroblastoma cells. Thus, in contrast to the situation with  $\rho^0$  cells derived from myoblasts, these neuroblastoma derived  $\rho^0$  cells apparently retain the ability to differentiate as judged by morphologic criteria (Herzberg, N. H. et al., Biochim. et Biophys. Acta, 1181:63-67 (1993)). This indicates that proteins encoded by the nuclear genes, essential to signal transduction and differentiation, are functional and not affected by EtBr treatment.

#### EXAMPLE XII

##### Preparation of AD and PD Cybrids

$\rho^0$  64/5 neuroblastoma cells were transformed with platelets from twelve Alzheimer's disease, three Parkinson disease and two age-matched control patients creating what are termed cybrid cells ( $\psi$ ). Platelets from AD and PD patients carrying mitochondria with single or multiple mutations in mtDNA encoding for ETC subunits, and from control (normal) patients, were isolated from 10 ml of whole blood drawn into an Becton Dickinson (Rutherford, NJ) Vacutainer containing anticoagulant (acid citrate dextrose). Samples of control SH-SY5Y cells were treated similarly. The samples were transferred into Accuspin tubes (Sigma)

over layers of histopaque (Sigma) and centrifuged for 10 minutes at 1000 x g at room temperature. The buffy coat containing both platelets and mononuclear lymphocytes was isolated, resuspended in five volumes of PBS and  
5 centrifuged at 1700 x g for 10 minutes, decanted and resuspended in DMEM with 5 mM EDTA (fusion medium).

Transformation was accomplished by a modification of Chomyn et al (Chomyn, A., et al., Am. J. Hum. Genet., 54:966-974 (1994)).  $\rho^0$  cells were removed from culture  
10 plates with trypsin, rinsed two times, and finally resuspended in fusion medium.  $\rho^0$  cells ( $4 \times 10^5$ , clone  $\rho^0$  64/5.0) were combined with platelets ( $1 \times 10^7$  platelets or  $1 \times 10^8$  platelets) in two mls of fusion medium and incubated 10 minutes at 37°C. Negative  
15 controls were  $\rho^0$  cells without added platelets and platelets without added  $\rho^0$  cells. The cell mixture was centrifuged at 300 x g for 10 minutes, resuspended in 57 ml of fusion medium. Polyethylene glycol (70% w/v PEG 1000, J.T. Baker, McGraw Park, Il) in fusion medium was  
20 added to the cells to achieve a final volume of 200 ml (final PEG concentration, 50%). Cells were incubated for 1.5 minutes at room temperature then diluted to a final volume of 10 mls with warm normal  $\rho^0$  cell medium and allowed to recover for 10 minutes at 37°C. The  
25 fused cells were plated in 75 cm<sup>2</sup> flasks. The medium was changed on following day.

The cells were allowed to recover in  $\rho^0$  medium for one week with medium changes every 2 days. Transformed cells (cybrids) repopulated with exogenous platelet  
30 mitochondria were selected by culturing in media lacking pyruvate and uridine with 10% dialyzed heat-inactivated FBS which removes residual uridine. These conditions were designed so that only repopulated cells could survive. The efficiency of transformation varied  
35 between 1 and 2% as judged by the number of surviving cells. Approximately  $1 \times 10^3$  fused cells were plated sparsely onto a 15 cm. tissue culture dish.



Isolated colonies appeared 4 to 6 weeks after the initial fusion. Based on the calculated reversion rates observed for clonal  $\rho^o$  64/5.0 ( $10^{-6}$ ) less than one spontaneously reverted clone should have appeared in these cultures. The aerobic phenotype is partially rescued by the transformation with mtDNA or mitochondria carrying mtDNA with specific mutations in the genes encoding for COX (disease phenotype).

Both the heterogeneous surviving cells (bulk phase) and isolated homogenous clones were propagated and assayed for complex I and IV activity and compared with complex I and IV activity from the parental SH-SY5Y cells (Table 7). The complex IV defect associated with brain and blood of Alzheimer's disease (Parker, W. D., et. al., Neurology, 40:1302-1303 (1990)) was successfully transferred to  $\rho^o$  64/5 neuroblastoma cells.

Table 7

## Complex IV Activity of Control and Alzheimer's Disease Cybrids

Cybrid	Patient Age	Bulk Cells	Complex IV min-mg <sup>-1</sup> (S.D.) % reduction	Clones
SH-SY5Y		2.025 (0.052)	-	
ψcon#0064	74	1.963 (0.010)	3.1	1.93 (0.73)
ψcon#0049	69	2.862 (0.070)	-41.3	2.49 (0.69)
ψAD#2330	83	1.500 (0.005)	25.9	1.72 (0.99)
"	week 1	1.762 (0.181)	25.8	
"	week 2	1.432 (0.078)	29.3	
ψAD#2418	66	1.520 (0.053)	24.9	1.75 (0.47)
ψAD#2490	85	1.375 (0.072)	32.1	1.28 (0.24)

The bulk phase of the three patients  $\psi$ AD#2330,  $\psi$ AD#2418 and  $\psi$ AD#2490 had lower complex IV specific activities. The average deficit was 27.8%. The complex I defect associated with the brain and blood of Parkinson's disease also was successfully transferred to  $\rho^0$  64/5 neuroblastoma cells. The average deficit was 44.5%. The age-matched control cybrids,  $\psi$ Con#0049 and  $\psi$ Con#0064, had normal complex I and IV activity. The clones isolated from the bulk phase had similar values, but showed a high degree of variation. This may reflect various degrees of heteroplasmy within the clones. The complex IV activities of the bulk phase for  $\psi$ AD#2418 were monitored for three weeks, consisting of three cell passages, and they continued to be stable. Thus the defect transferred appears to be stably maintained for at least three cell passages and is probably permanent.

Since the fused cells displayed a specific decrease in complex I and IV enzymatic activity that is characteristic of AD and PD neuronal cells, these procedures provide a cellular model (AD and PD cybrid cells) for further study of a major biochemical and genetic defect found in the blood and brain of AD and PD patients.

25

#### EXAMPLE XIII

##### Screening of Drugs and Treatments Using AD Cybrids

The AD cybrid cells constitute a new and unique cellular model system.

To use this system to screen for drugs that are potentially useful in treating AD, the AD cybrids are grown in the presence of agents known or suspected of having the ability to ameliorate the electron transport deficit in AD patients, or the cellular degeneration that apparently results from that deficit. Alternatively, screening can be done in a completely empirical manner, and compounds for screening can be

selected at random from those available anywhere in the world. Another alternative is to grow the cybrids in the presence of combinations of compounds, or subject them to other types of nutrients, vitamins, or other  
5 treatments.

After a period of treatment with a given compound, the treated cybrid cultures are tested to determine their COX activity relative to the COX activity of untreated cybrid control samples and normal cells, using  
10 methods such as those described hereinabove. In addition to measuring COX activity, treated and untreated cybrid controls observed microscopically to determine if the addition of the chemical agent has diminished the morphological changes characteristic of  
15 AD or PD. If treated cells exhibit an increase in COX activity and/or decrease in morphological degradation relative to untreated cybrids, the compound or compounds used in the treatment warrant further study to evaluate their potential effectiveness as drugs for treating AD.  
20 In addition, such positive results suggest that other similar chemical structures be screened for such activity.

107

## EXAMPLE XIV

## PD Cybrids

In a manner such as that described for construction of the AD cybrids, platelets from patients with Parkinson's disease and age-matched controls are fused with the  $\rho^0$  cells described above, creating PD cybrids. Clones of individual cybrids are then isolated as described above, and their Complex I activities are measured by methods described previously in this application.

Table 8

Comparison of Complex I and Complex IV Activity in Control and Parkinson's Disease Cybrids

15

	Cybrid	Complex I	
		nmol/min/mg	Complex IV sec/mg
	SH-SY5Y	28.2	0.120
	Control 1	27.7	0.135
20	Control 2	24.1	<u>0.154</u>
	Mean	26.7	0.136
	Parkinson's Disease 1	18.3	0.110
25	Parkinson's Disease 2	10.2	0.103
	Parkinson's Disease 3	15.9	<u>0.188</u>
	Mean	14.8	0.134

30

## EXAMPLE XV.

**Preparation of AD Cybrid Animals**

In another embodiment, mtDNA or mitochondria from diseased AD patients carrying specific multiple or  
5 single mutations in genes encoding for COX are introduced into animals, creating a mosaic animal.

A freshly fertilized mouse embryo, at about the 3 to 10 cell stage, is washed by saline lavage from the fallopian tubes of a pregnant mouse. Under a dissection  
10 microscope, the individual cells are teased apart, and are treated with ethidium bromide to induce a  $\rho^0$  state, in a manner such as that described hereinabove. Determining the appropriate duration and concentrations for ethidium bromide treatment may require the sacrifice  
15 of several embryos for Southern analysis to assure that mitochondrial function has been lost.

Then, cells so treated are re-populated with exogenous mitochondria isolated from the platelets of an AD affected patient, the preparation of which is  
20 described in Example XII above. One or more of the resulting cybrid cells are then implanted into the uterus of a pseudopregnant female by microinjection into the fallopian tubes. At the end of gestation, the COX activity of blood cells from one or more of the progeny  
25 is tested to confirm that the mitochondria behave as those of an AD patient. The presence of the AD COX gene defect can also be confirmed by DNA sequence analysis.

30

## EXAMPLE XVI

**Screening of Drugs and Treatments Using AD Cybrid Animals**

Known or unknown agents are delivered to the cybrid  
35 animals, and agents that rescue the disease phenotype or protect against the deleterious consequences associated with the disease phenotype are selected for further

study as potential drugs for the treatment of Alzheimer's Disease.

In addition, cells such as neurons and myoblasts can be isolated from these animals and used to screen for agents that rescue the disease phenotype or protect against the deleterious consequences associated with the disease phenotype. Such agents also should be further studied as potential treatments for Alzheimer's Disease.

10

## EXAMPLE XVII

## Diabetes

DNA Extraction From Blood Samples

Blood samples (7- 8 ml) from 14 NIDDM patients are collected in EDTA Vacutainer tubes (Scientific Products, Waukegan Park, IL). The blood samples are spun for 10 minutes at 2500 rpm at 4°C. The buffy coat containing white blood cells and platelets is removed. Five milliliters of TE buffer (10 mM Tris HCl, 1 mM EDTA, pH 7.5) are added to the buffy coat. This mixture is spun for 10 minutes at 2500 rpm and 4°C. The supernatant is removed and 5 ml of TE buffer, 200  $\mu$ l of 20% SDS and 100  $\mu$ l of proteinase K (400  $\mu$ g/ml final concentration) is added to the pellet. This mixture is incubated for 4 hours at 37°C with continuous shaking. DNA is extracted by 2 washes with phenol followed by two washes with chloroform: isoamyl alcohol (24:1). After each wash the solution is mixed, settled for 5 minutes and spun for 7 minutes at room temperature at 7000 rpm. The genomic DNA is precipitated by adding 1/10 volume of 3M sodium acetate and 2 volumes of 100% ethanol. The DNA is spun for 20 minutes at 4°C and the supernatant is removed. Ethanol (70%) is then added; the mixture is spun briefly and the supernatant is discarded. The dry pellet is resuspended in TE buffer and stored at 4 °C until use. The DNA is quantitated by  $A_{260}$  absorbance of a 1:50 dilution.

DNA Sequencing

The target cytochrome c oxidase gene sequences are amplified and cloned as described hereinabove in Example I. Plasmid DNA containing the COX gene inserts obtained  
5 as described in Example I is isolated using the Plasmid Quik™ Plasmid Purification Kit with Midi Columns (Qiagen, Chatsworth, CA). Plasmid DNA is purified from 35 ml bacterial cultures. The isolated DNA is resuspended in 100 µl TE buffer. The DNA is quantitated  
10 by A<sub>260</sub> absorbance of a 1:50 dilution.

Sequencing reactions using double stranded plasmid DNA are performed using the Prism™ Ready Reaction DyeDeoxy™ Terminator Cycle Sequencing Kit (Applied Biosystems Division, Perkin Elmer Corp., Foster City,  
15 CA). The DNA sequences are detected by fluorescence using the ABI 373A Automated DNA Sequencer (Applied Biosystems Division, Perkin Elmer Corp., Foster City, CA). For gene walking experiments, oligonucleotide primers are synthesized on the ABI 394 DNA/RNA  
20 Synthesizer using standard beta-cyanoethylphosphoramidite chemistry.

The following primer sequences are prepared from the published sequences of the COX genes for subunits I, II, and III:

25 COX1 primer 11 (5'-TGCTTCACTCAGCC-3');  
COX1 primer 1SF (5'-AGGCCTAACCCCTGTA-3');  
COX1 primer 11X (5'-AGTCCAATGCTTCACTCA-3');  
COX1 primer 12 (5'-GCTATAGTGGAGGC-3');  
COX1 primer 12A (5'-CTCCTACTCCTGCTCGCA-3');  
30 COX1 primer 12X (5'-TCCTGCTCGCATCTGCTA-3');  
COX1 primer 12XX (5'-CTCCTACTCCTGCTCGCA-3');  
COX1 primer 13 (5'-CCTACCAGGATTCG-3');  
COX1 primer 13A (5'-CCTACCAGGCTTCGGAA-3');  
COX1 primer 13X (5'-TCCTACCAGGCTTCGGAA-3');  
35 COX1 primer 14 (5'-CCTATCAATAGGAGC-3');  
COX1 primer 14XX (5'-GTCCTATCAATAGGAGCTGTA-3');  
COX1 primer 11C (5'-GTAGAGTGTGCAACC-3');



- COX1 primer 11CN (5'-GTCTACGGAGGCTCC-3');  
COX1 primer 11CX (5'-AGGTCTACGGAGGCTCCA-3');  
COX1 primer 11CXX (5'-AGGAGACACCTGCTAGGTGTA-3');  
COX1 primer 12C (5'-CCATACCTATGTATCC-3');  
5 COX1 primer 12CA (5'-TCACACGATAAACCCCTAGGAA-3');  
COX1 primer 12CX (5'-GACCATACCTATGTATCCAA-3');  
COX1 primer 13C (5'-CCTCCTATGATGGC-3');  
COX1 primer 13CN (5'-GTGTAGCCTGAGAATAGG-3');  
COX1 primer 13CXX (5'-GTCTAGGGTGTAGCCTGAGAA-3');  
10 COX1 primer 14C (5'-GGGTTCGATTCTTCC-3');  
COX1 primer 14CN (5'-TGGATTGAAACCAGC-3');  
COX1 primer 14CX (5'-GTTGGCTTGAAACCAGCTT-3');  
COX2 primer 21 (5'-TCATAACTTTGTCTGTC-3');  
COX2 primer 21N (5'-CATTTTCATAACTTTGTCTGTC-3');  
15 COX2 primer 21NA (5'-AGGTATTAGAAAAACCA-3');  
COX2 primer 21X (5'-TTCATAACTTTGTCTGTC-3');  
COX2 primer 2FSF (5'-AAGGTATTAGAAAAACC-3');  
COX2 primer 2SFA (5'-CCATGGCCTCCATGACTT-3');  
COX2 primer 22 (5'-TGGTACTGAACCTACG-3');  
20 COX2 primer 22A (5'-ACAGACGAGGTCAACGAT-3');  
COX2 primer 22X (5'-CATAACAGACGAGGTCAA-3');  
COX2 primer 21C (5'-AGTTGAAGATTAGTCC-3');  
COX2 primer 21CN (5'-TAGGAGTTGAAGATTAGTCC-3');  
COX2 primer 21CX (5'-TGAAGATAAGTCCGCCGTA-3');  
25 COX2 primer 22C (5'-GTTAATGCTAAGTTAGC-3');  
COX2 primer 22CXX (5'-AAGGTTAATGCTAAGTTAGCTT-3');  
COX3 primer 31 (5'-AAGCCTCTACCTGC-3');  
COX3 primer 31N (5'-CTTAATCCAAGCCTACG-3');  
COX3 primer 32 (5'-AACAGGCATCACCC-3');  
30 COX3 primer 32A (5'-CATCCGTATTACTCGCATCA-3');  
COX3 primer 31C (5'-GATGCGAGTAATACG-3');  
COX3 primer 31CX (5'-GATGCGAGTAATACGGAT-3');  
COX3 primer 32C (5'-AATTGGAAGTTAACGG-3');  
COX3 primer 32CX (5'-AATTGGAAGTTAACGGTA-3');  
35 COX3 primer 32CXX (5'-GTCAAACTAGTTAATTGGAA-3');

Sequencing reactions are performed according to the manufacturer's instructions. Electrophoresis and sequence analysis are performed using the ABI 373A Data Collection and Analysis Software and the Sequence Navigator Software (Applied Biosystems Division, Perkin Elmer Corp., Foster City, CA). Sequencing gels are prepared according to the manufacturer's specifications. An average of ten different clones from each individual is sequenced. The resulting COX sequences are aligned and compared with the published sequence. Differences in the derived sequence from the published sequence are noted and confirmed by sequence of the complementary DNA strand.

Five individuals with late onset diabetes had mutations in the genes encoding for COX subunit 1 and 2 (Table 9). These mutations are not seen in neurologic (Alzheimer's, Parkinson's, Huntington's, Diffuse Lewy Body, Senile Dementia of the Lewy Body type, Halvordan Spatz, Parasupranuclear Palsy, and other neurologic diseases) or aged controls.

Table 9

## Mitochondrial COX Mutations That Segregate to Late-Onset Diabetes

	Codon #			
	COX I		COX II	
	155	194	22	146
Normal amino acid	VAL	LEU	Thr	Ile
Normal DNA	GTC	CTA	ACC	ATT
Observed amino acid	ILE	Phe	Ile	Val
Mutant DNA	ATC	TTA	TCC	GTT
Patient				
KE	2	1	2	2
RI	5	4	3	3
DA	-	-	1	-
WO	-	2	-	-
PO	-	-	1	1

Numbers above indicate the number of times a given base change was observed in the ten clones that were sequenced for each diabetic patient. The base changes are differences in the observed sequence relative to the published sequence for human mitochondrial COX subunits. The codon number was determined from the beginning of the open reading frame of the 5'-end of the gene.

CLAIMS

We claim:

- 1 1. A method for detecting the presence of  
2 Alzheimer's disease in a subject, comprising the steps  
3 of:
  - 4 a) obtaining a biological sample  
5 containing mitochondria from said subject;  
6 and
  - 7 b) interrogating at least one mutation  
8 in the sequence of a mitochondrial cytochrome  
9 c oxidase gene which correlates with the  
10 presence of Alzheimer's disease.
- 1 2. A method according to claim 1 wherein at  
2 least one mutation exists between codon 155 and codon  
3 415 of the cytochrome c oxidase I gene.
- 1 3. A method according to claim 2 wherein at  
2 least one mutation in the cytochrome c oxidase I gene  
3 exists at a codon selected from the group consisting of  
4 codon 155, codon 167, codon 178, codon 193, codon 194,  
5 and codon 415.
- 1 4. A method according to claim 1 wherein at  
2 least one mutation exists between codon 20 and codon 150  
3 of the cytochrome c oxidase II gene.
- 1 5. A method according to claim 4 wherein at  
2 least one mutation in the cytochrome c oxidase II gene  
3 exists at a codon selected from the group consisting of  
4 codon 20, codon 22, codon 68, codon 71, codon 74, codon  
5 90, codon 95, codon 110, and codon 146.
- 1 6. A method according to claim 1 wherein the  
2 presence of at least one mutation in the sequence of a

3 mitochondrial cytochrome c oxidase gene is determined by  
4 hybridization with oligonucleotide probes.

1 7. A method according to claim 1 wherein the  
2 presence of at least one mutation in the sequence of a  
3 mitochondrial cytochrome c oxidase gene is determined  
4 using methods selected from the group of:

5 (a) methods based on the ligation of  
6 oligonucleotide sequences that anneal adjacent to one  
7 another on target nucleic acids;

8 (b) the polymerase chain reaction or  
9 variants thereof which depend on using sets of primers;  
10 and

11 (c) single nucleotide primer-guided  
12 extension assays.

1 8. A method according to claim 7 wherein the  
2 ligation method is the ligase chain reaction.

1 9. A method of according to claim 7 wherein of  
2 the sets of primers used, one is fully complementary and  
3 the other contains a mismatch.

1 10. A method according to claim 9 wherein the  
2 mismatch is either internal or at the 3' end of the sets  
3 of primers used.

1 11. A method according to claim 1 wherein said  
2 mitochondrial cytochrome c oxidase gene is amplified  
3 using a method selected from the group of PCR, RT-PCR  
4 and in vitro DNA replication.

1 12. The method of claim 1, wherein said mutation  
2 is interrogated by means of a probe comprising a  
3 nucleotide sequence complementary to either of the sense  
4 and anti-sense strands of a mitochondrial cytochrome c  
5 oxidase gene.

1 13. The method of claim 12, wherein said probe  
2 includes a region complementary to the sense and anti-  
3 sense strands of one or more codons selected from the  
4 group of:

5 (a) codon 155, codon 167, codon 178, codon  
6 193, codon 194, and codon 415 of the cytochrome c  
7 oxidase I gene; and  
8 (b) codon 20, codon 22, codon 68, codon 71,  
9 codon 74, codon 90, codon 95, codon 110, and codon 146  
10 of the cytochrome c oxidase II gene.

1 14. A method of detecting the genetic mutations  
2 which cause Alzheimer's disease, comprising the steps  
3 of:

4 a) determining the sequence of  
5 mitochondrial cytochrome c oxidase genes from  
6 subjects known to have Alzheimer's disease.  
7 b) comparing said sequence to that of  
8 known wild-type mitochondrial cytochrome c  
9 oxidase genes; and  
10 c) identifying recurrent mutations in  
11 said subjects.

1 15. The method of claim 14, wherein said known  
2 wild-type mitochondrial cytochrome c oxidase genes are  
3 selected from  
4 [SEQ ID NO 1]  
5 [SEQ ID NO 2], and  
6 [SEQ ID NO 3]

1 16. Isolated nucleotide sequences which  
2 correspond to or are complementary to portions of  
3 mitochondrial cytochrome c oxidase genes, wherein said  
4 sequences contain gene mutations which correlate with  
5 the presence of Alzheimer's disease.

1 17. The isolated nucleotide sequence of claim 16  
2 which contain gene mutations are COX I nucleotides 5964  
3 to 7505, COX II 7646 to 8329 or COX III nucleotides 9267  
4 to 10052.

1 18. The isolated nucleotide sequence of claim 16  
2 wherein said isolated sequences are labelled with a  
3 detectable agent.

1 19. The isolated nucleotide sequence of claim 16,  
2 wherein said detectable agent is selected from the group  
3 of radioisotopes, haptens, biotin, enzymes, fluorophores  
4 or chemilumiphores.

1 20. The isolated nucleotide sequence of claim 16,  
2 wherein said detectable agent is selected from the group  
3 of <sup>32</sup>P, digoxigenin, rhodamine, alkaline phosphatase,  
4 horseradish peroxidase, fluorescein and acridine.

1 21. A method for inhibiting the transcription or  
2 translation of mutant cytochrome c oxidase encoding  
3 genes, comprising the steps of:

4 a) contacting said genes with antisense  
5 sequences which are specific to said mutant  
6 sequences; and

7 b) allowing hybridization between said  
8 target mutant cytochrome c oxidase gene and  
9 said antisense sequences under conditions  
10 under which said antisense sequences bind to  
11 and inhibit transcription or translation of  
12 said target mutant cytochrome c oxidase genes  
13 without preventing transcription or  
14 translation of wild-type cytochrome c oxidase  
15 genes.

1 22. The method of claim 21 wherein Alzheimer's  
2 disease or diabetes mellitus is treated and wherein said

3 cytochrome c oxidase genes contain mutations at one or  
4 more codons selected from the group of:

5 (a) codon 155, codon 167, codon 178, codon  
6 193, codon 194, and codon 415 of the cytochrome c  
7 oxidase I gene; and

8 (b) codon 20, codon 22, codon 68, codon 71,  
9 codon 74, codon 90, codon 95, codon 110, and codon 146  
10 of the cytochrome c oxidase II gene.

1 23. A probe for detection of a disease state  
2 associated with one or more mutations in mitochondrial  
3 cytochrome c oxidase genes comprising a nucleotide  
4 sequence complementary to either of the sense and anti-  
5 sense strands of said one or more mutations in said  
6 mitochondrial cytochrome c oxidase genes.

1 24. The probe of claim 23 wherein said probe  
2 includes a region complementary to the sense and anti-  
3 sense strands of one or more codons selected from the  
4 group of:  
5 (a) codon 155, codon 167, codon 178, codon  
6 193, codon 194, and codon 415 of the cytochrome c  
7 oxidase I gene; and  
8 (b) codon 20, codon 22, codon 68, codon 71,  
9 codon 74, codon 90, codon 95, codon 110, and codon 146  
10 of the cytochrome c oxidase II gene.

1 25. A kit comprising a probe for detection of an  
2 Alzheimer's disease or diabetes mellitus genotype, said  
3 probe comprising a nucleotide sequence complementary to  
4 either of the sense and anti-sense strands of a  
5 mitochondrial cytochrome c oxidase gene.

1 26. The kit of claim 28, wherein said probe  
2 includes a region complementary to the sense and anti-  
3 sense strands of one or more codons selected from the  
4 group of:



5 (a) codon 155, codon 167, codon 178, codon  
6 193, codon 194, and codon 415 of the cytochrome c  
7 oxidase I gene; and

8 (b) codon 20, codon 22, codon 68, codon 71,  
9 codon 74, codon 90, codon 95, codon 110, and codon 146  
10 of the cytochrome c oxidase II gene.

1 27. A therapeutic composition comprising  
2 antisense sequences which are specific to mutant  
3 cytochrome c oxidase genes or mutant messenger RNA  
4 transcribed therefrom, said antisense sequences adapted  
5 to bind to and inhibit transcription or translation of  
6 said target mutant cytochrome c oxidase genes without  
7 preventing transcription or translation of wild-type  
8 cytochrome c oxidase genes.

1 28. The therapeutic composition of claim 27,  
2 wherein a disease selected from the group of Alzheimer's  
3 disease and diabetes mellitus is treated and wherein  
4 said cytochrome c oxidase genes contain mutations at one  
5 or more codons selected from the group of:

6 (a) codon 155, codon 167, codon 178, codon  
7 193, codon 194, and codon 415 of the cytochrome c  
8 oxidase I gene; and

9 (b) codon 20, codon 22, codon 68, codon 71,  
10 codon 74, codon 90, codon 95, codon 110, and codon 146  
11 of the cytochrome c oxidase II gene.

1 29. A method for detecting the presence of a  
2 disease of mitochondrial origin in a subject, comprising  
3 the steps of:

4 a) obtaining a biological sample  
5 containing mitochondria from said subject;  
6 and

7 b) interrogating at least one variant  
8 polypeptide, arising from one or more  
9 mutations in one or more subunits of

10 mitochondrial cytochrome c oxidase genes,  
11 which correlates with the presence of said  
12 disease.

1 30. The method of claim 29, wherein said disease  
2 is selected from the group of Alzheimer's disease and  
3 diabetes mellitus and said mutation is interrogated  
4 using monoclonal antibodies or polyclonal antibodies.

1 31. A ribozyme adapted to hybridize to and cleave  
2 mitochondrial mRNA molecules that encode for mutant  
3 cytochrome c oxidase subunits.

1 32. A method for selectively introducing a  
2 conjugate molecule into mitochondria with defective  
3 cytochrome c oxidase genes comprising:  
4 a) providing a conjugate molecule that is  
5 selectively introduced into said mutated mitochondria,  
6 said conjugate molecule comprising a targeting molecule  
7 conjugated to a toxin or to an imaging ligand by a  
8 linker; and  
9 b) contacting said mutant mitochondria with  
10 said conjugate molecule.

1 33. The method of claim 32, wherein said  
2 targeting molecule is a lipophilic cation selected from  
3 the group consisting of acridine orange derivatives and  
4 JC-1 derivatives.

1 34. The method of claim 32, wherein said linker  
2 contains a functional group selected from ester, ether,  
3 thioether, phosphorodiester, thiophosphorodiester,  
4 carbonate, carbamate, hydrazone, oxime, amino and amide.

1 35. The method of claim 32, wherein said  
2 targeting molecule and linker comprise a 10-N-(R<sub>1</sub>-X)-3,6-  
3 bis(dimethylamino)acridine derivative wherein R<sub>1</sub> is an

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4 aliphatic group containing from 5 to 20 carbons, and X  
5 is attached to the terminal carbon of the alkane group  
6 and is selected from the group of ester, ether,  
7 thioether, phosphorodiester, thiophosphorodiester,  
8 carbonate, carbamate, hydrazone, oxime, amino and amide.

1 36. The method of claim 32, wherein said  
2 targeting molecule is selected from the group consisting  
3 of derivatives of rhodamine 123 and JC-1.

1 37. The method of claim 32, wherein said target  
2 molecule is a JC-1 derivative, and wherein said linker  
3 comprises a group selected from ester, ether, thioether,  
4 phosphorodiester, thiophosphorodiester, carbonate,  
5 carbamate, hydrazone, oxime, amino and amide.

1 38. The method of claim 37 wherein the linker is  
2 attached to said JC-1 derivative via substitution of at  
3 least one of the four chlorine atoms at the 5, 5', 6 and  
4 6' carbon positions of the JC-1 derivative.

1 39. The method of claim 37, wherein said linker  
2 is attached to the JC-1 derivative via substitution of  
3 the terminal carbon hydrogen of at least one of the four  
4 ethyl groups at the 1,1',3 and 3' positions of the JC-1  
5 derivative.

1 40. The method of claim 37, wherein said linker  
2 is attached to the JC-1 derivative via substitution of  
3 one of the olefinic hydrogens of the JC-1 derivative.

1 41. The method of claim 37, wherein said linker  
2 further comprises an alkyl group of 2-20 carbon atoms.

1 42. The method of claim 32 wherein said imaging  
2 ligand is selected from the group of radioisotopes,

3   haptens, biotin, enzymes, fluorophores or  
4   chemilumiphores.

1   43.           The method of claim 40 wherein said toxin is  
2   selected from phosphate, thiophosphate, dinitrophenol  
3   and maleimide and antisense oligonucleic acids.

1   44.           An immortal  $\rho^{\circ}$  cell line.

1   45.           The immortal  $\rho^{\circ}$  cell line of claim 44, wherein  
2   said cell line is a  $\rho^{\circ}$  form of an immortal neural cell  
3   line.

1   46.           The immortal  $\rho^{\circ}$  cell line of claim 44 wherein  
2   said cell line is undifferentiated.

1   47.           The undifferentiated immortal  $\rho^{\circ}$  cell line of  
2   claim 46 wherein said cell line is capable of being  
3   induced to differentiate.

1   48.           The immortal  $\rho^{\circ}$  cell line of claim 47, wherein  
2   said cell line is a  $\rho^{\circ}$  form of a neuroblastoma cell line.

1   49.           The  $\rho^{\circ}$  cell line of claim 48, wherein said  
2   cell line is a  $\rho^{\circ}$  form of neuroblastoma cell line SH-  
3   SY5Y.

1   50.           A cybrid cell line, comprising: cultured  
2   immortal cells having genomic and mitochondrial DNAs of  
3   differing biological origins.

1   51.           The cybrid cell line of claim 50, wherein  
2   said genomic DNA has its origin in an immortal  $\rho^{\circ}$  cell  
3   line, and said mitochondrial DNA has its origin in a  
4   human tissue sample.

1 52. The cybrid cell line of claim 50, wherein  
2 said genomic DNA has its origin in an undifferentiated  
3 immortal  $\rho^0$  cell line that is capable of being induced to  
4 differentiate, and said mitochondrial DNA has its origin  
5 in a human tissue sample.

1 53. The cybrid cell line of claim 52, wherein  
2 said undifferentiated immortal  $\rho^0$  cell line is a  $\rho^0$  form  
3 of a neuroblastoma cell line.

1 54. The cybrid cell line of claim 53, wherein  
2 said neuroblastoma cell line is derived from the  
3 neuroblastoma cell line SH-SY5Y.

1 55. The cybrid cell line of claim 51, wherein  
2 said human tissue sample is derived from a patient  
3 having a disease that is associated with mitochondrial  
4 defects.

1 56. The cybrid cell line of claim 52, wherein  
2 said human tissue sample is derived from a patient  
3 having a disease that is associated with mitochondrial  
4 defects.

1 57. The cybrid cell line of claim 52, wherein  
2 said undifferentiated immortal  $\rho^0$  cell line is a  $\rho^0$  form  
3 of a neuroblastoma cell line and said human tissue  
4 sample is derived from a patient having a neurological  
5 disease that is associated with mitochondrial defects.

1 58. The cybrid cell line of claim 51 wherein said  
2 human tissue sample is from a patient having a disorder  
3 selected from the group consisting of Alzheimer's  
4 Disease, Parkinson's Disease, Huntington's disease,  
5 dystonia, Leber's hereditary optic neuropathy,  
6 schizophrenia, myoclonic-epilepsy-lactic-acidosis -and-

7 stroke (MELAS), and myoclonic-epilepsy-ragged-red-  
8 fiber --syndrome (MERRF).

1 59. The cybrid cell line of claim 52, wherein  
2 said undifferentiated immortal  $\rho^0$  cell line is a  $\rho^0$  form  
3 of neuroblastoma cell line SH-SY5Y and said human tissue  
4 sample is from a patient having a disorder selected from  
5 the group consisting of Alzheimer's Disease, Parkinson's  
6 Disease, Huntington's disease, dystonia, Leber's  
7 hereditary optic neuropathy, schizophrenia,  
8 mitochondrial encephalopathy-lactic-acidosis -and-stroke  
9 (MELAS), and myoclonic-epilepsy-ragged-red-fiber --  
10 syndrome (MERRF).

1 60. The cybrid cell line of claim 52, wherein  
2 said undifferentiated immortal  $\rho^0$  cell line is a  $\rho^0$  form  
3 of neuroblastoma cell line SH-SY5Y and said human tissue  
4 sample is from a patient having Alzheimer's Disease.

1 61. A differentiated cybrid cell line resulting  
2 from induction of differentiation in cells of the cybrid  
3 cell line of claim 52.

1 62. A method of constructing a cybrid cell line,  
2 comprising the steps of:  
3 a.) treating an immortal cell line with a  
4 chemical agent capable of irreversibly  
5 disabling mitochondrial electron  
6 transport and thus converting said cell  
7 line into an immortal  $\rho^0$  cell line; and  
8 b.) transfecting said immortal  $\rho^0$  cell line  
9 with isolated mitochondria to form said  
10 cybrid cell line.

1 63. The method of claim 62, wherein said immortal  
2 cell line is undifferentiated, but capable of being  
3 induced to differentiate.

1 64. The method of claim 62, wherein said isolated  
2 mitochondria are purified from a patient known to be  
3 afflicted with a disorder associated with a  
4 mitochondrial defect.

1 65. The method of claim 62, wherein said chemical  
2 agent is ethidium bromide.

1 66. A method of constructing cybrid cell lines,  
2 comprising the steps of:

3 a.) treating an immortal neuroblastoma cell  
4 line with ethidium bromide to irreversibly disable  
5 mitochondrial electron transport and thus convert said  
6 cell line into an immortal  $\rho^0$  neuroblastoma cell line;  
7 and

8 b.) transfecting said immortal  $\rho^0$   
9 neuroblastoma cell line with mitochondria isolated from  
10 tissue of a patient afflicted with a disorder selected  
11 from the group consisting of Alzheimer's Disease,  
12 Parkinson's Disease, Huntington's disease, dystonia,  
13 Leber's hereditary optic neuropathy, schizophrenia,  
14 myoclonic-epilepsy-lactic-acidosis -and-stroke (MELAS),  
15 and myoclonic-epilepsy-ragged-red-fiber --syndrome  
16 (MERRF), to form said cybrid cell line.

1 67. A method for evaluating a compound for  
2 potential utility in the treatment of a disorder that is  
3 associated with mitochondrial defects, comprising the  
4 steps of:

5 a.) contacting a predetermined quantity of  
6 the test compound with cultured immortal cybrid cells  
7 having genomic DNA originating from an immortal  $\rho^0$  cell  
8 line and mitochondrial DNA originating from tissue of a  
9 patient having a disease that is associated with  
10 mitochondrial defects; and

11                   b.) measuring a phenotypic trait in said  
12 cybrid cells that is affected by said mitochondrial  
13 defect; and

14                   c.) establishing whether and to what extent  
15 said drug is capable of causing said trait to become  
16 more similar to those of control cells having  
17 mitochondria that lack said defect, which capability  
18 indicates that the compound has potential utility in the  
19 treatment of said disorder.

1   68. A method for evaluating a compound for potential  
2 utility in the treatment of a disorder that is  
3 associated with mitochondrial defects according to claim  
4 67, comprising the steps of:

5                   a.) inducing the differentiation of cultured  
6 undifferentiated immortal cybrid cells having genomic  
7 DNA originating from an immortal  $\rho^0$  cell line and  
8 mitochondrial DNA originating from tissue of a patient  
9 having a disease that is associated with mitochondrial  
10 defects; and

11                   b.) contacting a predetermined quantity of  
12 the test compound with said differentiated cybrid cells;  
13 and

14                   c.) measuring a phenotypic trait in said  
15 differentiated cybrid cells that is affected by said  
16 mitochondrial defect; and

17                   d.) establishing whether and to what extent  
18 said drug is capable of causing said trait to become  
19 more similar to those of control cells having  
20 mitochondria that lack said defect, which capability  
21 indicates that the compound has potential utility in the  
22 treatment of said disorder.

1   69. A method for the diagnosis of disorders that are  
2 associated with mitochondrial defects, comprising the  
3 steps of:



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- 4           a.) obtaining from a patient a biological  
5 sample containing mitochondria; and  
6           b.) transferring said mitochondria into  
7 immortal  $\rho^0$  cells to form cybrid cells; and  
8           c.) measuring a phenotypic trait in said  
9 cybrid cells that is caused by the mitochondrial defect  
10 associated with the disorder or disorders being tested  
11 for; and  
12           d.) establishing whether said cybrid cells  
13 exhibit said trait as do cells of patients suffering  
14 from said disorder, which indicates the presence of the  
15 disorder in said patient.

1   70. A method for the diagnosis of disorders that are  
2 associated with mitochondrial defects according to claim  
3 69, comprising the steps of:

- 4           a.) obtaining from a patient a biological  
5 sample containing mitochondria; and  
6           b.) transferring said mitochondria into  
7 undifferentiated immortal  $\rho^0$  cells to form cybrid cells;  
8 and  
9           c.) inducing said cybrid cells to  
10 differentiate; and  
11           d.) measuring one or more phenotypic trait in  
12 said differentiated cybrid cells that is caused by the  
13 mitochondrial defect associated with the disorder or  
14 disorders being tested for; and  
15           e.) establishing whether said cybrid cells  
16 exhibit said trait as do cells of patients suffering  
17 from said disorder, which indicates the presence of the  
18 disorder in said patient.

1   71. A cybrid animal comprising: a multicellular,  
2 non-human animal, having genomic and mitochondrial DNAs  
3 of differing biological origins.

1 72. A method of preparing a cybrid animal,  
2 comprising the steps of:  
3 a.) isolating embryonic cells from a  
4 multicellular, non-human animal; and  
5 b.) treating said embryonic cells with a  
6 chemical agent capable of irreversibly disabling  
7 mitochondrial electron transport, thus converting said  
8 cells to a  $\rho^0$  state; and  
9 c.) transfecting said immortal  $\rho^0$  cell line  
10 with mitochondria isolated from another cell source, to  
11 produce said cybrid animal.

1 73. A method for evaluating a compound for potential  
2 utility in the treatment of a disorder that is  
3 associated with mitochondrial defects, comprising the  
4 steps of:  
5 a.) contacting a predetermined quantity of  
6 the test compound with a cybrid animal of claim 71; and  
7 b.) measuring or observing one or more  
8 phenotypic trait in said cybrid animal that is affected  
9 by said mitochondrial defect; and  
10 c.) establishing whether and to what extent  
11 said drug is capable of causing said trait or traits to  
12 become more similar to those of control animals having  
13 mitochondria that lack said defect, which capability  
14 indicates that said compound has potential utility in  
15 the treatment of said disorder.

1 74. A cybrid cell line, comprising: cultured  
2 cells having genomic and mitochondrial nucleic acids of  
3 differing biological origins, wherein either the  
4 mitochondrial or the genomic nucleic acid is derived  
5 from an individual exhibiting symptoms of late onset  
6 diabetes mellitus or at risk for developing symptoms for  
7 late onset diabetes mellitus.

1 75. The cybrid cell line of claim 74, wherein  
2 said cybrid is made by:  
3 a.) treating a parental cell or cell line  
4 with a chemical agent capable of converting said cell or  
5 cell line into a  $\rho^0$  cell line; and  
6 b.) transfecting said  $\rho^0$  cell line with  
7 isolated mitochondria to form said cybrid cell line.

1 76. The cybrid of claim 75, wherein said parental  
2 cell or cell line is undifferentiated, but capable of  
3 being induced to differentiate.

1 77. The cybrid of claim 75, wherein said cybrid  
2 cell line is immortal.

1 78. The cybrid of claim 77, wherein cybrid cell  
2 line is undifferentiated, but capable of being induced  
3 to differentiate.

1 79. A cybrid cell line according to claim 75,  
2 wherein the parental cell or cell line is selected from  
3 the group consisting of: a zygote, an embryonic cell  
4 capable of differentiating and giving rise to a tissue  
5 or an individual, a germ cell line, a pancreatic  $\beta$  cell  
6 or cell line, a fat cell or cell line, a muscle cell or  
7 cell line, and an insulin-responsive cell other than a  
8 pancreatic  $\beta$  cell line, a fat cell, or a muscle cell.

1 80. A method for evaluating a compound for utility in  
2 the diagnosis or treatment of diabetes mellitus, said  
3 method comprising:

4 a.) contacting a predetermined quantity of  
5 said compound with cultured cybrid cells having genomic  
6 DNA originating from a  $\rho^0$  cell line and mitochondrial DNA  
7 originating from tissue of a human having a disorder  
8 that is associated with late onset diabetes mellitus;  
9 and

10                   b.) measuring a phenotypic trait in said  
11 cybrid cells that is affected by said mitochondrial  
12 defect.

1   81.           A method according to claim 80, wherein the  $\rho^0$   
2 cell line is immortal.

1   82.           A method for evaluating a compound for its  
2 utility in the diagnosis and treatment of diabetes  
3 mellitus, said method comprising:

4                   a.) inducing the differentiation of cultured  
5 undifferentiated cybrid cells having genomic DNA  
6 originating from a  $\rho^0$  cell line and mitochondrial DNA  
7 originating from tissue of a human having a disorder  
8 that is associated with late onset diabetes mellitus;  
9 and

10                  b.) contacting a predetermined quantity of  
11 said compound with said differentiated cybrid cells; and  
12                  c.) measuring a phenotypic trait in said  
13 differentiated cybrid cells that is affected by said  
14 mitochondrial defect.

1   83.           A method according to claim 82, wherein said  
2  $\rho^0$  cell line is immortal.

1   84.           A method for detecting the presence of a  
2 human disease of mitochondrial origin comprising:

3                   a) obtaining a biological sample containing  
4 mitochondria from said human; and

5                   b) determining the presence of at least one  
6 mitochondrial mutation or gene which correlates with the  
7 disease.

1   85.           A method according to claim 84 wherein said  
2 at least one mitochondrial mutation or gene is a  
3 mutation in a cytochrome c oxidase gene.

1 86. A method according to claim 85 wherein the  
2 disease is selected from Alzheimer's disease and  
3 diabetes mellitus.

1 87. A method according to claim 86 wherein said  
2 mutation in a cytochrome c oxidase gene is at one or  
3 more codons selected from the group of codon 155, codon  
4 167, codon 178, codon 193, codon 194, and codon 415 of  
5 the cytochrome c oxidase I gene and codon 20, codon 22,  
6 codon 68, codon 71, codon 74, codon 90, codon 95, codon  
7 110, and codon 146 of the cytochrome c oxidase II gene.

1 88. An isolated nucleotide sequence which is at  
2 least partially complementary to a mitochondrial DNA  
3 sequence containing at least one mutation which  
4 correlates with the presence of a human disease of  
5 mitochondrial origin.

1 89. The isolated nucleotide sequence of claim 88,  
2 wherein said mitochondrial DNA sequence contains at  
3 least one mutation selected from the group consisting of  
4 mutations in COX I nucleotides 5964 to 7505, and COX II  
5 nucleotides 7646 to 8329

1 90. The isolated nucleotide sequence of claim  
2 88, wherein said human disease of mitochondrial origin  
3 is selected from diabetes mellitus and Alzheimer's  
4 disease.

1 91. The isolated nucleotide sequence of claim 90,  
2 wherein said mitochondrial DNA sequence contains at  
3 least one mutation selected from the group consisting of  
4 mutations between codon 155 and codon 415 in the  
5 cytochrome c oxidase I gene and codon 20 and codon 146  
6 in the cytochrome c oxidase II gene.

1 92. The isolated nucleotide sequence of claim 91,  
2 wherein said mitochondrial DNA sequence contains at  
3 least one mutation found at a codon selected from the  
4 group consisting of codon 155, codon 167, codon 178,  
5 codon 193, codon 194, and codon 415 of the cytochrome c  
6 oxidase I gene and codon 20, codon 22, codon 68, codon  
7 71, codon 74, codon 90, codon 95, codon 110, and codon  
8 146 of the cytochrome c oxidase II gene.

1 93. A method of inhibiting the transcription or  
2 translation of one or more mutant cytochrome c oxidase-  
3 encoding nucleic acids comprising:  
4 a) contacting said gene or genes with  
5 antisense sequences specific to said mutant sequence or  
6 sequences; and  
7 b) allowing hybridization between said target  
8 mutant cytochrome c oxidase gene or genes and said  
9 antisense sequence or sequences.

1 94. A method according to claim 93, wherein  
2 hybridization is performed under conditions wherein the  
3 antisense sequence or sequences bind to and inhibit  
4 transcription or translation of said target mutant  
5 cytochrome c oxidase gene or genes without preventing  
6 transcription or translation of wild-type cytochrome c  
7 oxidase genes or other mitochondrial genes.

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FIGURE 1

COX I 5'-END NON-CODING REGION, CODING REGION:5964-7505 (1542bp), and  
3'-END NON-CODING REGION  
(SEQ. ID. NO. 1)

5'-AGAGGCCTAA CCCCTGTCTTTAGATTTTAC AGTCCAATGCTTCACTCAGC  
CATTTTACCT CACCCCCACT G

ATG TTC GCC GAC CGT TGA CTA TTC TCT ACA AAC CAC AAA GAC ATT GGA ACA  
CTA TAC CTA TTA TTC GGC GCA TGA GCT GGA GTC CTA GGC ACA GCT CTA AGC  
CTC CTT ATT CGA GCC GAG CTG GGC CAG CCA GGC AAC CTT CTA GGT AAC GAC  
CAC ATC TAC AAC GTT ATC GTC ACA GCC CAT GCA TTT GTA ATA ATC TTC TTC  
ATA GTA ATA CCC ATC ATA ATC GGA GGC TTT GGC AAC TGA CTA GTT CCC CTA  
ATA ATC GGT GCC CCC GAT ATG GCG TTT CCC CGC ATA AAC AAC ATA AGC TTC  
TGA CTC TTA CCT CCC TCT CTC CTA CTC CTG CTC GCA TCT GCT ATA GTG GAG  
GCC GGA GCA GGA ACA GGT TGA ACA GTC TAC CCT CCC TTA GCA GGG AAC TAC  
TCC CAC CCT GGA GCC TCC GTA GAC CTA ACC ATC TTC TCC TTA CAC CTA GCA  
GGT GTC TCC TCT ATC TTA GGG GCC ATC AAT TTC ATC ACA ACA ATT ATC AAT  
ATA AAA CCC CCT GCC ATA ACC CAA TAC CAA ACG CCC CTC TTC GTC TGA TCC  
GTC CTA ATC ACA GCA GTC CTA CTT CTC CTA TCT CTC CCA GTC CTA GCT GCT  
GGC ATC ACT ATA CTA CTA ACA GAC CGC AAC CTC AAC ACC ACC TTC TTC GAC  
CCC GCC GGA GGA GGA GAC CCC ATT CTA TAC CAA CAC CTA TTC TGA TTT TTC  
GGT CAC CCT GAA GTT TAT ATT CTT ATC CTA CCA GGC TTC GGA ATA ATC TCC  
CAT ATT GTA ACT TAC TAC TCC GGA AAA AAA GAA CCA TTT GGA TAC ATA GGT  
ATG GTC TGA GCT ATG ATA TCA ATT GGA TTC CTA GGG TTT ATC GTG TGA GCA  
CAC CAT ATA TTT ACA GTA GGA ATA GAC GTA GAC ACA CGA GCA TAT TTC ACC  
TCC GCT ACC ATA ATC ATC GCT ATC CCC ACC GGC GTC AAA GTA TTT AGC TGA  
CTC GCC ACA CTC CAC GGA AGC AAT ATG AAA TGA TCT GCT GCA GTG CTC TGA  
GCC CTA GGA TTC ATC m CTT TTC ACC GTA GGT GGC CTG ACT GGC ATT GTA  
TTA GCA AAC TCA TCA CTA GAC ATC GTA CTA CAC GAC ACG TAC TAC GTT GTA  
GCC CAC TTC CAC TAT GTC CTA TCA ATA GGA GCT GTA TTT GCC ATC ATA GGA  
GGC TTC ATT CAC TGA TTT CCC CTA TTC TCA GGC TAC ACC CTA GAC CAA ACC  
TAC GCC AAA ATC CAT TTC ACT ATC ATA TTC ATC GGC GTA AAT CTA ACT TTC  
TTC CCA CAA CAC TTT CTC GGC CTA TCC GGA ATG CCC CGA CGT TAC TCG GAC  
TAC CCC GAT GCA TAC ACC ACA TGA AAC ATC CTA TCA TCT GTA GGC TCA TTC  
ATT TCT CTA ACA GCA GTA ATA TTA ATA ATT TTC ATG ATT TGA GAA GCC TTC  
GCT TCG AAG CGA AAA GTC CTA ATA GTA GAA GAA CCC TCC ATA AAC CTG GAG  
TGA CTA TAT GGA TGC CCC CCA CCC TAC CAC ACA TTC GAA GAA CCC GTA TAC  
ATA AAA TCT AGA

CAAAAAAGGA AGGAATCGAA CCC CCCAAAG CTGGTTTCAA GCCAACCCCA  
TGGCCTCCAT GACTTTTTC AAAAAAGTATT AGAAAAACCA TTTCATAACT TTGTCAAAGT  
TAAATTATAG GCTAA-3'

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## FIGURE 2

COX II 5'-END NON-CODING REGION, CODING REGION:7646-8329(684bp), AND  
3'-END NON-CODING REGION

(SEQ. ID. NO. 2)

5'-AGGTATTAGA AAAACCATTT CATAACTTTG TCGTCAAAGT TAAATTATAG  
GCTAAATCCT ATATATCTTA

ATG GCA CAT GCA GCG CAA GTA GGT CTA CAA GAC GCT ACT TCC CCT ATC ATA  
GAA GAG CTT ATC ACC TTT CAT GAT CAC GCC CTC ATA ATC ATT TTC CTT ATC  
TGC TTC CTA GTC CTG TAT GCC CTT TTC CTA ACA CTC ACA ACA AAA CTA ACT  
AAT ACT AAC ATC TCA GAC GCT CAG GAA ATA GAA ACC GTC TGA ACT ATC CTG  
CCC GCC ATC ATC CTA GTC CTC ATC GCC CTC CCA TCC CTA CGC ATC CTT TAC  
ATA ACA GAC GAG GTC AAC GAT CCC TCC CTT ACC ATC AAA TCA ATT GGC CAC  
CAA TGG TAC TGA ACC TAC GAG TAC ACC GAC TAC GGC GGA CTA ATC TTC AAC  
TCC TAC ATA CTT CCC CCA TTA TTC CTA GAA CCA GGC GAC CTG CGA CTC CTT  
GAC GTT GAC AAT CGA GTA GTA CTC CCG ATT GAA GCC CCC ATT CGT ATA ATA  
ATT ACA TCA CAA GAC GTC TTG CAC TCA TGA GCT GTC CCC ACA TTA GGC TTA  
AAA ACA GAT GCA ATT CCC GGA CGT CTA AAC CAA ACC ACT TTC ACC GCT ACA  
CGA CCG GGG GTA TAC TAC GGT CAA TGC TCT GAA ATC TGT GGA GCA AAC CAC  
AGT TTC ATG CCC ATC GTC CTA GAA TTA ATT CCC CTA AAA ATC TTT GAA ATA  
GGG CCC GTA TTT ACC CTA TAG

CACCCCCTCT ACCCCCTCTA GAGCCCACTG TAAAGCTAAC TTAGCATTAA C  
CTTTTAAGT TAAAGATTAA GAGAACCAAC ACCTGTTTAC AGTGAAATGC-3'



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## FIGURE 3

COS III 5' - END NON-CODING REGION, CODING REGION: 9267-10052 (785 bp), AND 3'-END NON-CODING REGION  
(SEQ. ID. NO. 3)

5'-TCGCTGTCGC CTTAATCCAA GCCTACGTTT TCACACTTCT AGTAAGCCTC  
TACCTGCACG ACAACACATA

ATG ACC CAC CAA TCA CAT GCC TAT CAT ATA GTA AAA CCC AGC CCA TGA CCC  
CTA ACA GGG GCC CTC TCA GCC CTC CTA ATG ACC TCC GGC CTA GCC ATG TGA  
TTT CAC TTC CAC TCC ATA ACG CTC CTC ATA CTA GGC CTA CTA ACC AAC ACA  
CTA ACC ATA TAC CAA TGA TGG CGC GAT GTA ACA CGA GAA AGC ACA TAC CAA  
GGC CAC CAC ACA CCA CCT GTC CAA AAA GGC CTT CGA TAC GGG ATA ATC CTA  
TTT ATT ACC TCA GAA GTT TTT TTC TTC GCA GGA TTT TTC TGA GCC TTT TAC  
CAC TCC AGC CTA GCC CCT ACC CCC CAA TTA GGA GGG CAC TGG CCC CGA ACA  
GGC ATC ACC CCG CTA AAT CCC CTA GAA GTC CCA CTC CTA AAC ACA TCC GTA  
TTA CTC GCA TCA GGA GTA TCA ATC ACC TGA GCT CAC CAT AGT CTA ATA GAA  
AAC AAC CGA AAC CAA ATA ATT CAA GCA CTG CTT ATT ACA ATT TTA CTG GGT  
CTC TAT TTT ACC CTC CT ACAA GCC TCA GAG TAC TTC GAG TCT CCC TTC ACC  
ATT TCC GAC GGC ATC TAC GGC TCA ACA TTT TTT GTA GCC ACA GGC TTC CAC  
GGA CTT CAC GTC ATT ATT GGC TCA ACT TTC CTC ACT ATC TGC TTC ATC CGC  
CAA CTA ATA TTT CAC TTT ACA TCC AAA CAT CAC TTT GGC TTC GAA GCC GCC  
GCC TGA TAC TGG CAT TTT GTA GAT GTG GTT TGA CTA TTT CTG TAT GTC TCC  
ATC TAT TGA TGA GGG TCT TAC

TCTTTTAGTA TAAATAGTAC CGTTAACTTC CAATT  
AACTA GTTTTGACAA CATTCAAAAA AGAGTAATAA ACTTCGCCTT AATTTTAATA  
ATCAACACCC-3'

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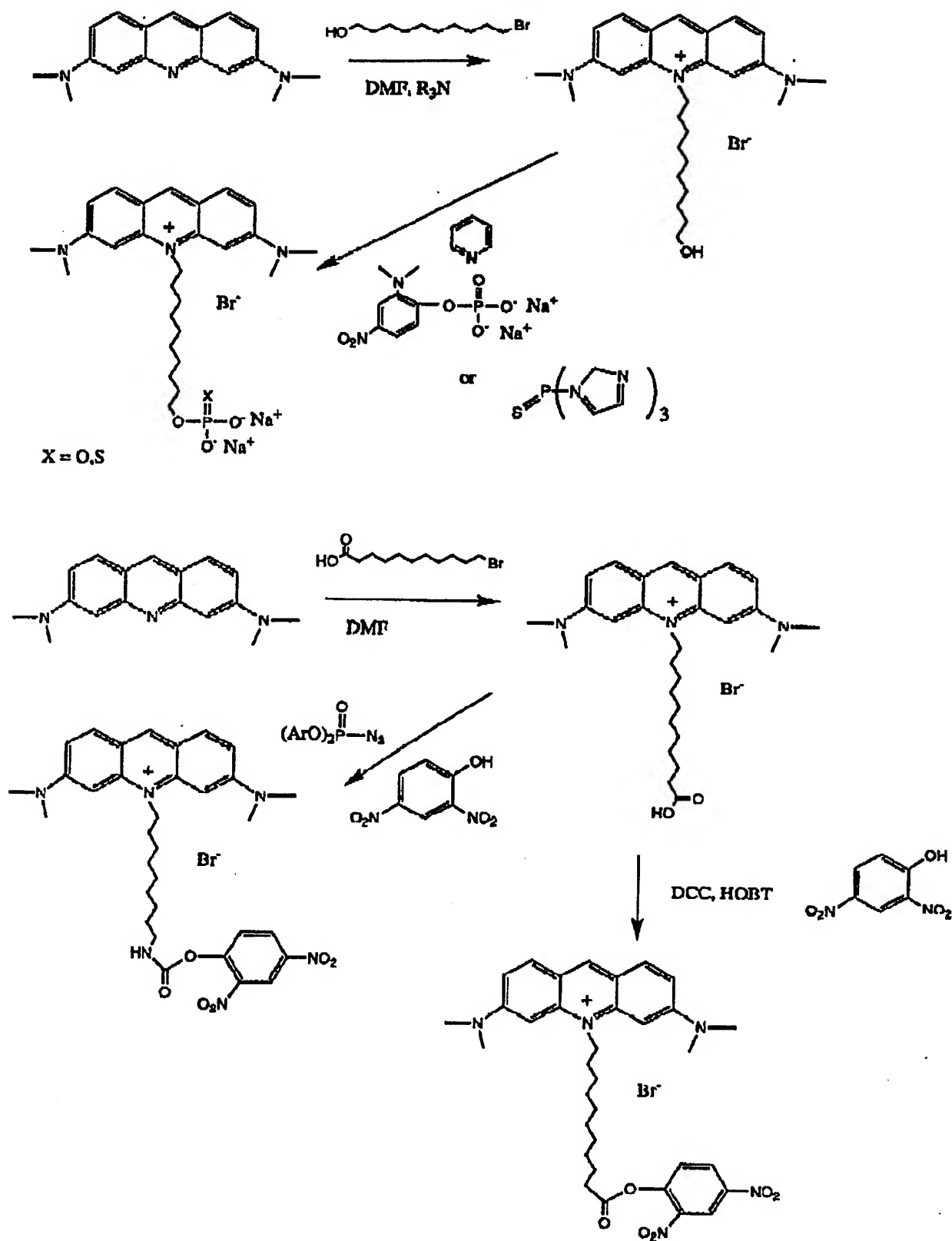
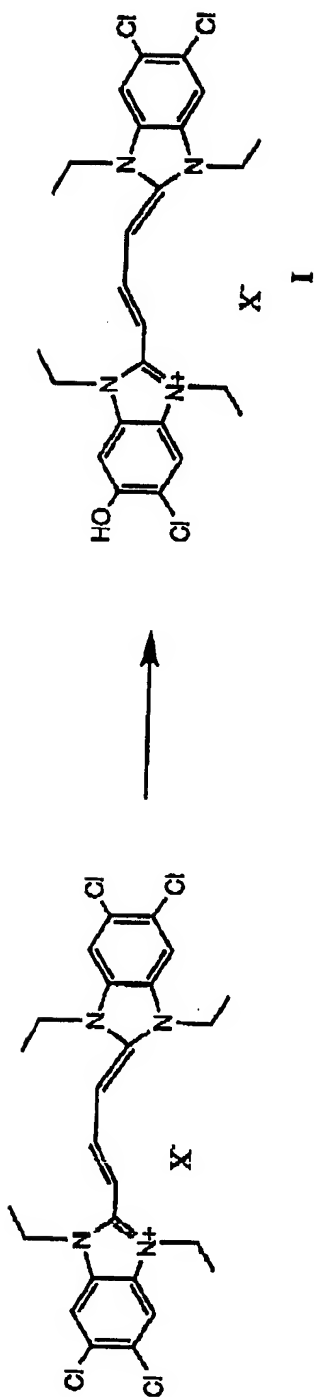


Fig. 4

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Converting chloro to heteroatom to provide attachment points for drug molecules  
 Heteroatom could be O, N, S

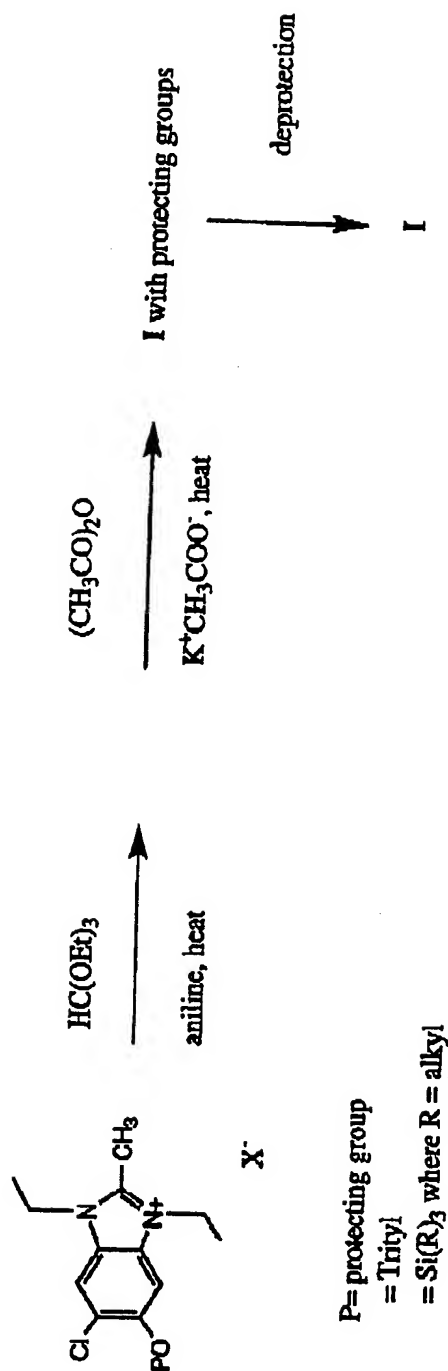


Fig. 5

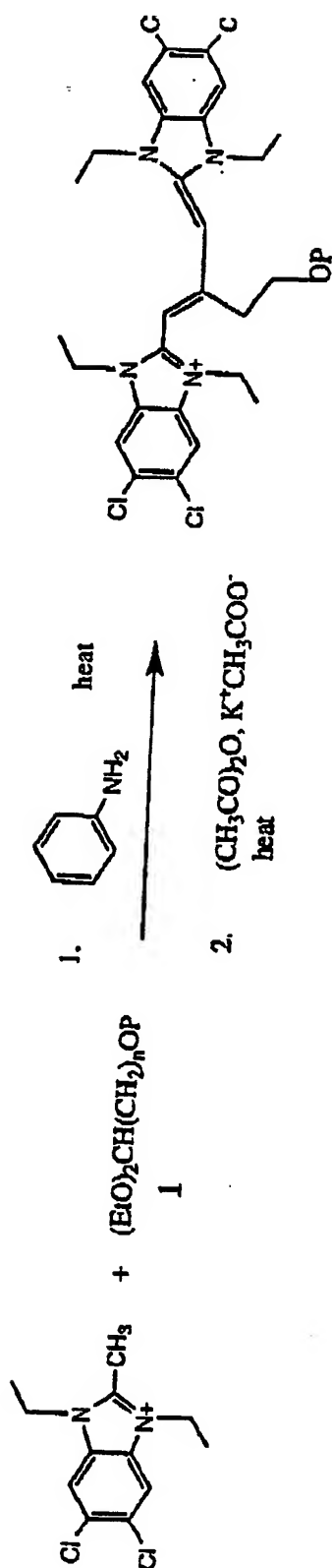


Fig. 6

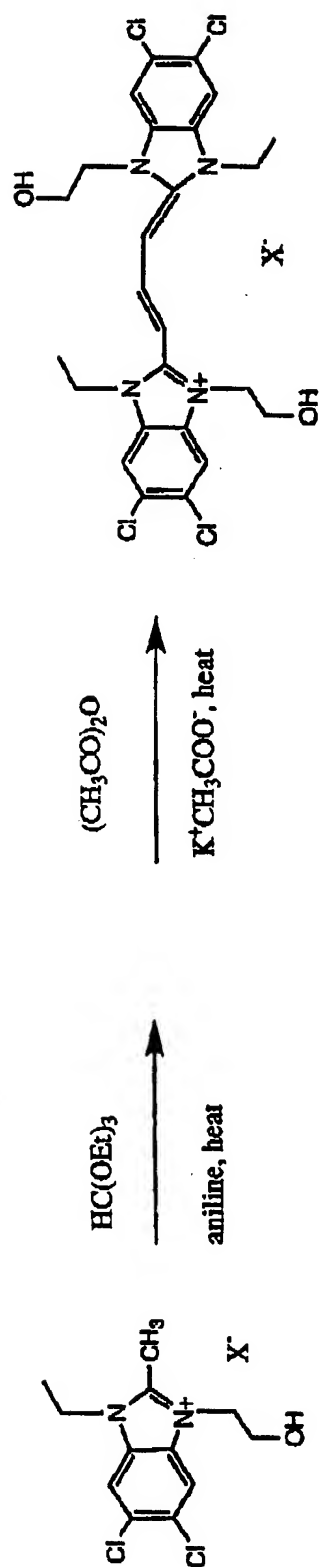
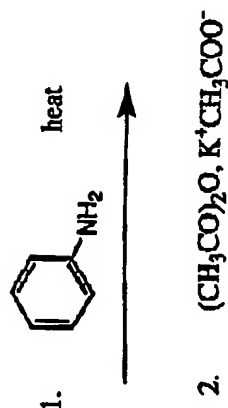
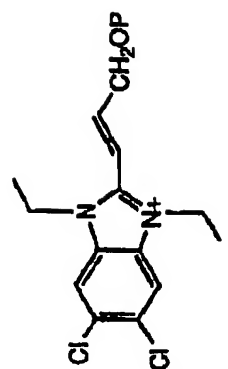


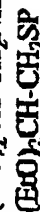
Fig. 7

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P = protecting group  
=  $\text{Si}(\text{R})_3$  where R = alkyl  
= Trryl

other possible linking units:



n = 2-10

x = OH

=  $\text{NH}_2$

= SH

= COOH



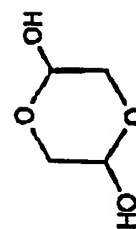
n = 2-10

x = OH

=  $\text{NH}_2$

= SH

= COOH



For each acetal represented, this could also be a ketal where H is replaced by an alkyl group C1 to C10

Fig. 8

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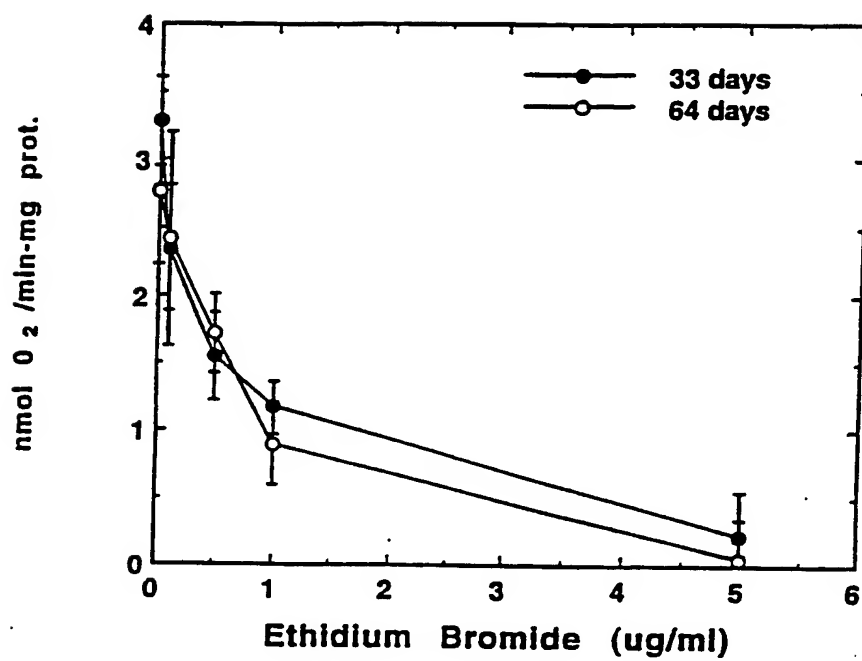


Fig. 9

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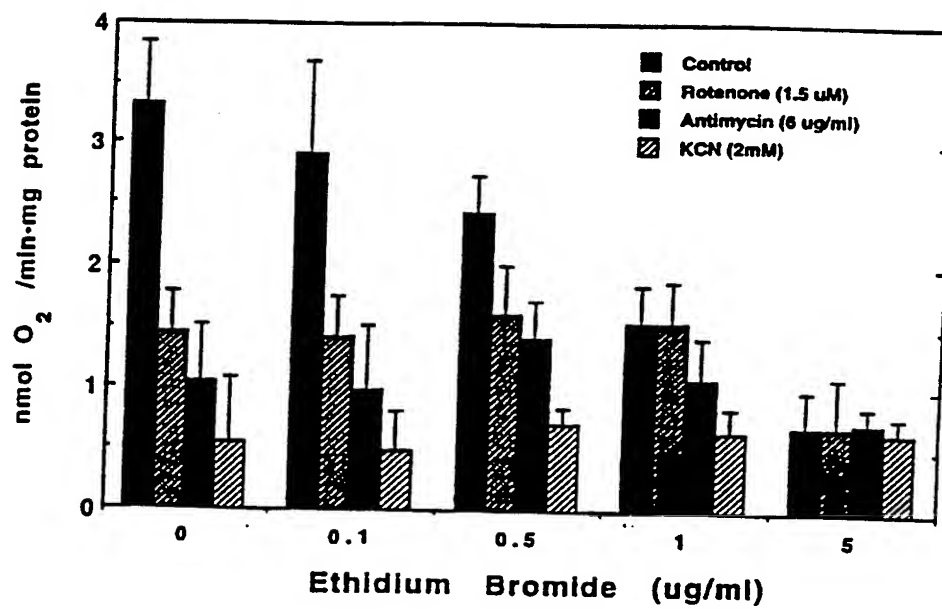


Fig. 10



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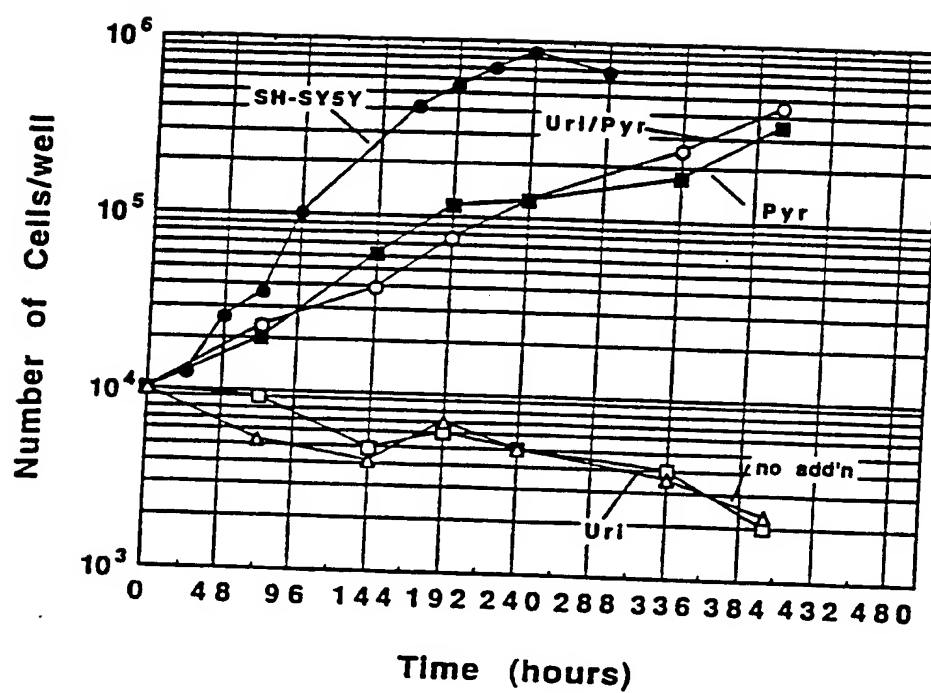


Fig. 11

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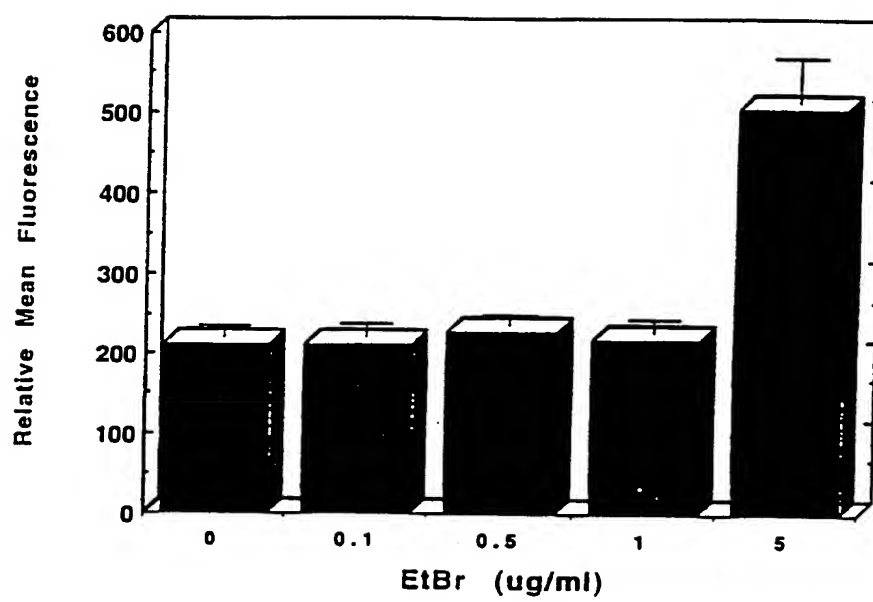


Fig. 12

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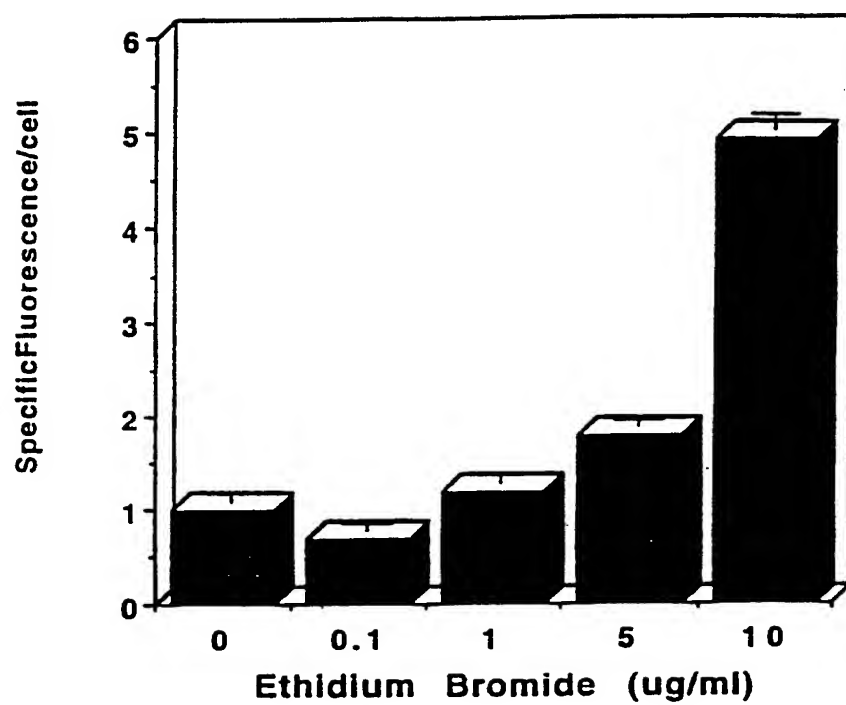


Fig. 13

## INTERNATIONAL SEARCH REPORT

International application No.  
PCT/US95/04063

**A. CLASSIFICATION OF SUBJECT MATTER**

IPC(6) : Please See Extra Sheet.

US CL : Please See Extra Sheet.

According to International Patent Classification (IPC) or to both national classification and IPC

**B. FIELDS SEARCHED**

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 536/24.1, 24.3, 24.5; 435/6, 69.1, 91.3, 172.2, 240.27; 530/350, 401; 514/44; 800/2

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS, MEDLINE, HCA, EMBASE, BIOSIS, SCISEARCH, LIFESCI

**C. DOCUMENTS CONSIDERED TO BE RELEVANT**

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	JOURNAL OF GERIATRIC PSYCHIATRY AND NEUROLOGY, Vol. 5, issued April-June 1992, Bennet et al., "Cytochrome oxidase inhibition: a novel animal model of Alzheimer's disease," pages 93-101, see entire document.	1-94
Y	HUMAN GENETICS, Vol. 88, issued 1991, Marzuki et al., "Normal variants of human mitochondrial DNA and translation products: the building of a reference data base," pages 139-145, see entire document.	1-94

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

* Special categories of cited documents:	"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A" document defining the general state of the art which is not considered to be of particular relevance	"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E" earlier document published on or after the international filing date	"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&" document member of the same patent family
"O" document referring to an oral disclosure, use, exhibition or other means	
"P" document published prior to the international filing date but later than the priority date claimed	

Date of the actual completion of the international search 26 JUNE 1995	Date of mailing of the international search report <b>13 JUL 1995</b>
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. (703) 305-3230	Authorized officer <i>D. Fruse</i> SCOTT HOUTTEMAN Telephone No. (703) 308-0196

# INTERNATIONAL SEARCH REPORT

International application No.  
PCT/US95/04063

## Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:  
because they relate to subject matter not required to be searched by this Authority, namely:
2. ☐ Claims Nos.:  
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:
3. ☐ Claims Nos.:  
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

## Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

Please See Extra Sheet.

1. ☒ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest



The additional search fees were accompanied by the applicant's protest.



No protest accompanied the payment of additional search fees.

**A. CLASSIFICATION OF SUBJECT MATTER:**  
IPC (6):

C07H 21/04; C12N 5/12, 5/16, 5/22, 15/00, 15/07; C12P 21/00; C12Q 1/68; C07K 14/00, 16/18, 16/44; A61K 48/00

**A. CLASSIFICATION OF SUBJECT MATTER:**  
US CL :

536/24.1, 24.3, 24.5; 435/6, 69.1, 91.3, 172.2, 240.27; 530/350, 401; 514/44; 800/2

**BOX II. OBSERVATIONS WHERE UNITY OF INVENTION WAS LACKING**

This ISA found multiple inventions as follows:

This application contains the following inventions or groups of inventions which are not so linked as to form a single inventive concept under PCT Rule 13.1. In order for all inventions to be examined, the appropriate additional examination fees must be paid. Note, some claims are generic to two groups and are listed in both groups.

Group I, claims 1-20, 23-30 and 88-92, drawn to isolated cytochrome C nucleotide sequences, probes, kits and therapeutic compositions.

Group II, claims 21, 22, 93 and 94, drawn to methods of transcription inhibition.

Group III, claims 29 and 30 drawn to methods of detection of mitochondrial originating diabetes.

Group IV, claim 31, drawn to a ribozyme.

Group V, claims 32-43, drawn to methods of conjugate molecule introduction.

Group VI, claims 44-49, drawn to immortal p<sup>0</sup> cell lines.

Group VII, claims 50-61, drawn to cybrid cell lines.

Group VIII, claims 62-66, drawn to methods of constructing cybrid cell lines.

Group IX, claims 67-68 and 80-83 drawn to methods of evaluating compounds for treatment utility.

Group X, claims 69 and 70, drawn to methods of diagnosis of mitochondrial associated defects.

Group XI, claims 71 and 72, drawn to cybrid non-human animals.

Group XII, claim 73, drawn to a method of compound evaluation comprising animal treatment.

Group XIII, claims 50-61 and 74-79, drawn to cybrid cell lines from individuals with diabetes.

Group XIV, claims 84-87, drawn to methods for detecting mitochondria originating disease comprising determining the presence of a mutation or gene.

The first product and first method of use have been included into group I. The special technical feature of the group I products and methods is the cytochrome c mutation that correlates with Alzheimer's disease. Groups II-XIII represent other products and other methods of use of these other products. The inventions listed as Groups I-XIV do not relate to a single inventive concept under PCT Rule 13.1 because, under PCT Rule 13.2, they lack the same or corresponding special technical features for the following reasons:

Groups II-III, V, VIII-X, XII and XIV are drawn to methods. These methods contain different steps, different reagents and result in different end products and so these methods do not share a special technical feature with each other or with the methods of group I.

Groups IV, VI, VII, XI and XIII are drawn to products which are different from the group I product, cytochrome c mutations that correlate with Alzheimer's disease. Therefore, the products of groups IV, VI, VII, XI and XIII do not share a special technical feature with the products of group I.

The methods of groups II-III, V, VIII-X, XII and XIV do not use the products of groups IV, VI, VII, XI and XIII. Therefore, these methods do not share a special technical feature with these products.